THE UNIVERSITY
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The Origins and Phenotypic Heterogeneity of Macrophages in Endometriosis

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Declaration

I hereby declare that all work carried out during this PhD was performed by myself, unless otherwise stated, under the supervision of Dr Erin Greaves, Professor Andrew Horne and Professor Jeff Pollard. This thesis has not previously been submitted for any other degree or qualification.

Chloe Elizabeth Hogg Date: 08/10/2019

Acknowledgements

I would like to start by thanking my supervisors, Dr Erin Greaves, Professor Andrew Horne and Professor Jeff Pollard. I've learnt so much during my time as a PhD student and I'd like to thank you for your dedication to my development as a scientist and for offering me so many amazing opportunities to travel and share my research with others. I've come away from my PhD with a so many new skills and acquired knowledge, so thank you.

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Table of Contents

Abstract

Endometriosis is a chronic inflammatory disorder associated with pelvic pain and infertility that affects ~176 million reproductive age women worldwide. It is defined by the presence of endometrial-like tissue outside the uterus (lesions). The aetiology of endometriosis is not fully understood however we know that menstrual effluent is refluxed into the peritoneal cavity during menstruation in 90% of women, and it is hypothesised that in some women this tissue can adhere to form lesions (Sampsons theory). Current management of endometriosis is unsatisfactory and there is an unmet need for new treatments. Macrophages play a key role in the growth, vascularisation and innervation of endometriosis lesions. We know from other diseases such as cancer that macrophages with differential origins and phenotypes play distinct roles in disease development, however our current knowledge of macrophage origins, recruitment pathways and phenotype in endometriosis is limited. In this thesis I aimed to define the origin of lesion-resident macrophages, the recruitment pathways active in disease and assess lesion macrophage phenotypic heterogeneity using the Edinburgh mouse model of endometriosis. This mouse model mimics the retrograde reflux of menses material by stimulating an artificial menstruation-like event in mice, whereby this tissue is collected and injected into the peritoneal cavity of recipient mice. I hypothesised that lesion resident macrophages have multiple origins, that CCL2 is an important chemokine for macrophage recruitment and that multiple macrophage populations exist within endometriosis lesions that have differential phenotypic profiles.

I used Csf1r-EGFP mice (MacGreen; macrophages express green fluorescent protein) to characterise the origins of lesion resident macrophages. Endometriosis was induced in WT mice using donor endometrial tissue from Csf1r-EGFP mice. 16% of lesion macrophages were GFP+ endometrial-derived macrophages and the remaining 84% were GFP- infiltrating macrophages of recipient origin. To assess infiltration of tissue-resident large peritoneal macrophages (LPM) and monocyte-derived small peritoneal macrophages (SPM) into lesions, I adoptively transferred LPM or SPM (isolated from Csf1r-EGFP mice) into the peritoneal cavity of endometriosis mice. GFP+ LPM infiltrated lesions however GFP+ SPM were not identified within lesion tissue. Using Ly6C, F4/80 dual immunofluorescence I identified both Ly6C+ monocytes and Ly6C+ F4/80+ monocyte-derived macrophages in lesions.

Mice with endometriosis exhibited a ~3 fold increase of peritoneal macrophages expressing CCR2 (receptor for chemokine (C-C) motif ligand 2 (CCL2)) compared to sham controls and 53% of lesion resident macrophages expressed CCR2. To assess the importance of CCR2 in the development of endometriosis lesions, endometriosis was induced in CCR2 knockout mice compared to WT. CCR2

knockout mice developed a similar number of endometriosis lesions to WT mice and exhibited an influx of Ly6C+ monocytes into the peritoneal cavity, which also infiltrated into lesions. Notably, endometriosis lesions from CCR2 knockout animals had fewer F4/80+ macrophages. CCL2 knockout mice developed more endometriosis lesions than WT and demonstrated similar infiltration of Ly6C+ monocytes and F4/80+ macrophages into lesion tissue compared to WT. Importantly, CCL2 knockout mice had an increase in CD45+ leukocytes within the peritoneal cavity which could have attributed to the difference observed in lesion number.

Initially, I performed flow cytometry analysis on lesion tissue to assess phenotypic heterogeneity and demonstrated that multiple macrophage populations were present, based on differential expression of CCR2, MHC II, CX3CR1 and CD86. I also observed that endometrial macrophage phenotypic heterogeneity was lost upon incorporation into lesions, suggesting macrophage reprogramming upon incorporation into the lesion microenvironment. To gain further granularity into the phenotypic heterogeneity of lesion macrophages, using single-cell RNA-sequencing, I performed unbiased transcriptional profiling of CD45+ cells in lesions (6006 cells), donor endometrial tissue (1306 cells) and peritoneal lavage cells from sham (5645 cells) and endometriosis mice (6720 cells). Clustering analysis identified 5 lesion-resident macrophage populations that had differential transcriptomic profiles. Comparison of lesion-resident macrophage populations with macrophage clusters from endometrial tissue and the peritoneum confirmed that lesions contained macrophages from different origins.

Collectively, these results indicate that lesion resident macrophages are derived from multiple origins and that 5 different macrophage populations exist within lesions that have differential transcriptomic profiles. Macrophage/monocyte recruitment may be in part mediated by the CCL2/CCR2 signalling pathway but when this pathway is disrupted in our model of endometriosis there appears to be compensation such that monocytes are still recruited to the peritoneal cavity and lesions. Herein, I provide an important insight into macrophage biology in endometriosis and provide a platform for further studies that aim to identify disease-specific macrophage populations that could potentially be targeted for clinical benefit.

Lay Summary

Endometriosis is a common disease that affects 1 in 10 women of reproductive age. Endometriosis is characterized by the growth of the lining of the uterus (the endometrium) outside the uterus, usually within the abdominal cavity. The growth of these endometrial-like tissue deposits (lesions) is associated with chronic pelvic pain and infertility. We don't fully understand why the disease occurs, however a popular theory is that menstrual material that is re-gurgitated into the abdominal cavity through the fallopian tubes during menstruation, adheres to the lining of the abdominal cavity to form lesions. Current treatment options for endometriosis are ineffective and new therapies are desperately needed. Immune cells are involved in regulating the health of our tissues, however in some disease states they can become dysregulated, thus contributing to disease. In endometriosis, immune cells called macrophages encourage the growth and development of endometriosis lesions. We know from other diseases, such as cancer, that macrophages can contribute to disease, and that targeting dysregulated macrophages could provide benefit for patients. However, current knowledge about macrophages in the context of endometriosis is limited, and more research is required to understand their role and complexity in disease.

In this thesis my aims were to understand where macrophages within endometriosis lesions come from (their origins), to investigate how they are recruited to lesions, and to assess whether multiple macrophage populations exist which could potentially play different roles in the disease. To answer these questions I used a mouse model of endometriosis. This model uses a hormonal schedule which mirrors the human menstrual cycle, triggering an artificial menstruation-like event in the mice. This menses-like tissue is collected and injected into the abdominal cavity of recipient mice, which go on to develop human-like endometriosis lesions.

I found, using tracing experiments where macrophages were fluorescently labelled, that macrophages within lesions were derived from three different origins. Macrophages from donor endometrial tissue made up 16% of the lesion macrophage population and the remaining 84% of macrophages were derived from macrophages that had infiltrated into the tissue (from the recipient). I characterised that these infiltrating populations consisted of macrophages from the abdominal cavity and from precursor cells in the blood (monocyte-derived macrophages).

Macrophages respond to signals known as chemokines. I was interested in a specific chemokine known as chemokine (C-C motif) ligand 2 (CCL2) because it is the main chemokine used by monocytes and macrophages to move into tissues. I demonstrated that the receptor for CCL2 (CCR2) was expressed by 53% of lesion macrophages

and that mice with endometriosis also had increased levels of macrophages that expressed CCR2 in the abdominal cavity.

To study how macrophages are attracted to endometriosis lesions I used mice that were genetically modified such that the genes encoding CCR2 or CCL2 were deleted from the their genome. Endometriosis lesions from CCR2 knockout mice had a similar number of macrophage precursor cells (monocytes) but a reduced number of macrophages compared to unmodified mice (wild type; WT), suggesting that CCR2 is required for macrophage but not monocyte recruitment to lesions. CCL2 knockout mice developed endometriosis lesions which had a similar number of both monocytes and macrophages compared to WT. These experiments show that whilst CCL2 may be important for recruitment of monocytes and macrophages in our mouse model, when it is knocked out, another chemokine is able to compensate for this loss and still cause the recruitment of monocytes and macrophages to lesions. These results do indicate however, that signalling through CCR2, perhaps by another chemokine which binds to CCR2, does appear to be important for the recruitment of macrophages to lesions. Overall, a number of different chemokines may be able to cause the movement of monocytes and macrophages into lesions.

Many different ‘types’ of macrophages exist and they can modify the functions they perform based on local signals in the tissue where they reside. By analysing the genetic material of single cells to determine what genes they express I could demonstrate that there are five different ‘types’ of macrophages present in endometriosis lesions. This information suggests that these different ‘types’ of macrophages could play different roles in endometriosis, however further research is required to confirm this.

In summary, I have demonstrated that endometriosis lesion resident macrophages have differential origins, can be recruited via chemokines / receptors other than CCL2/ CCR2 and that there are multiple ‘types’ of macrophages present in lesions. This research provides an important platform for future studies and reveals that macrophage biology in endometriosis is more complex than previous research suggests.

Publications

Hogg C, Horne AW, Greaves E. Endometriosis-Associated Macrophages: Origin, Phenotype and Function. *Frontiers in Endocrinology*. 2020: https://doi.org/10.3389/fendo.2020.00007

Horne AW, Ahmad FS, Carter R, Simitsidellis I, Greaves E, **Hogg C**, Morton NM, Saunders PTK. Repurposing dichloroacetate for the treatment of women with endometriosis. *PNAS.* 2019; 10.1073/pnas.1916144116

Forster R, Sarginson L, Velichkova A, **Hogg C**, Dorning A, Horne AW, Saunders PT, Greaves E. Macrophage-derived insulin-like growth factor-1 is a key neurotrophic and sensitizing factor in endometriosis. *FASEB J.* 2019; https://doi.org/10.1096/fj.201900797R

Hogg C, Panir K, Henlon Y, Dhami P, Rosser M, Mack M, Vrljicak P, Soong D, Pollard JW, Ott S, Jenkins SJ, Horne AW, Greaves E. Monocyte-derived macrophages are guardians of the peritoneal cavity in a mouse model of induced endometriosis. (Manuscript prepared)

Dorning A, **Hogg C**, Horne, AW, Greaves E. Variability amongst end-point analyses in mouse models of endometriosis; a study utilizing non-invasive *in vivo* bioluminescent monitoring of lesions. (In preparation)

Presentations and Awards

The Society for Reproductive Investigation: Targeting Inflammation to Improve Reproductive Health Across the Life Course Symposium — University of Edinburgh -16th – 18th August 2017 -Oral –2nd prize

ESHRE Campus Course, Methodological Approaches for Investigating Endometrial Function and Endometriosis - Edinburgh Radisson Blu Hotel - 18th-19th August 2017 – Poster

MOMENDO EU Exchange Programme Meeting – University of Edinburgh – 20th September 2017 - Oral

Barbour Watson Trust Grant - £2000 – Project entitled ‘Deciphering Phenotypic Heterogeneity of the Endometriosis Associated Macrophage’– October 2017

Royal College of Obstetricians and Gynaecologists Annual Academic Meeting – 9th – 10th Feb 2018 -Royal College of Obstetricians and Gynaecologists, London – Poster and ‘poster platform’ oral -2nd prize

Endometriosis Network Meeting – University of Liverpool, Liverpool Women’s Hospital – 19th April 2018 - Oral

A Cell for All Seasons: Macrophage Satellite Symposium – University of Edinburgh – Tuesday 1st May 2018 – Oral – 1st prize

A Cell for all seasons: Macrophages in Health and Disease 4th Annual Symposium– University of Edinburgh – Wednesday 2nd May 2018 – Oral (invited to present in main session after winning 1st prize at the satellite meeting)

The British Society for Immunology: Edinburgh Immunology Group Summer Symposium – 12th June 2018 – University of Edinburgh - Poster

The Society for the Study of Reproduction 2018 Annual Conference – Pathways to Discovery: Signals for Reproduction, Development and Longevity – New Orleans, Louisiana - 10th-13th July 2018 – Poster and 'platform poster' oral

Society for Reproduction and Fertility - £800 travel grant to attend the Society for the Study of Reproduction 51st Annual Meeting – New Orleans – July 2018

The Society for Reproductive Investigation 66th Annual Scientific Meeting – Paris, France – 12th-16th March 2019 – Poster

The British Society for Immunology, Inflammation: from Initiation to Restoration Conference – University of Edinburgh - 24th - 26th April - Poster

The Society for the Study of Reproduction 52nd Annual Conference, Beyond Possible: Remarkable Transformation of Reproductive Biology – San Jose, California – 18th-21st July –Oral

The World Endometriosis Society - Young Scientist Journalist at the Society for the Study of Reproduction 52nd Annual Conference - Article published on the World Endometriosis Society website - July 2019

Courses Attended

Edinburgh Cellular Genomics Consortium: Single Cell Sequencing Workshop – University of Edinburgh - 4th December 2017

EMBL Course: Single Cell RNA Sequencing – The European Molecular Biology Laboratory, Heidelberg - 15th-18th May 2018

Abstracts from Thesis

Submitted for:

- The Society for Reproductive Investigation: Targeting Inflammation to Improve Reproductive Health Across the Life Course Symposium - University of Edinburgh -16th – 18th August 2017
- ESHRE Campus Course, Methodological Approaches for Investigating Endometrial Function and Endometriosis - Edinburgh Radisson Blu Hotel - 18th-19th August 2017

Abstract:

Peritoneal macrophage dynamics in a mouse model of endometriosis.

Chloe Hogg, Andrew W Horne, Jeffrey Pollard, Erin Greaves

MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, Scotland

Introduction: Endometriosis is a chronic inflammatory disorder characterised by the presence of endometrial-like tissue (lesions) outside the uterus, most commonly in the peritoneal cavity. Macrophages are critical for growth and angiogenesis in endometriosis lesions, however little is known about the origins or activation status of these cells. In the peritoneal cavity macrophages exist as two populations; large peritoneal macrophages (LPM; high in number and associated with immunosurveillance and homeostasis) and small peritoneal macrophages (SPM; usually low in number but increase with inflammation). We aimed to discern whether mice with induced endometriosis have altered numbers of peritoneal macrophages and whether these subsets are incorporated into endometriosis lesions.

Methods: Using our established mouse model of endometriosis (including naïve (n=8) and sham+E2 (n=6) controls for comparison), peritoneal lavage using 7ml DMEM was performed and peritoneal macrophage subsets were analysed by flow cytometry using F4/80 (LPM) and MHC II (SPM) expression, to delineate macrophage populations. Dual immunofluorescence was performed on mouse lesions using large and small peritoneal macrophage markers, GATA6 and F4/80, and RELMα and MHC II, respectively.

Results: Flow cytometry revealed that there was no significant difference in LPM between sham+E2 (10554 cells/µl) compared to naïve mice (5382 cells/µl; p=0.097). Mice with endometriosis had higher numbers of LPM than naïve mice but lower numbers than sham mice (7299 cells/µl). A similar non-significant trend was observed with SPM. Heterogeneity in numbers and ratios of LPM and SPM in endometriosis mice was seen with some animals exhibiting an increased SPM: LPM ratio. Immunofluorescence identified that 48% (±SEM 4%) of cells in endometriosis lesions express F4/80 (n=10 lesions from n=7 animals), and of these 2% (±SEM 1%) co-express GATA6.

Conclusions: Heterogeneity in the numbers and ratios of LPM and SPM was observed in endometriosis mice. We speculate that lesion number, distribution and

relative activity of lesions could contribute to this heterogeneity. Our data also suggests that large peritoneal macrophages do not significantly contribute to the lesion-resident macrophage population.

Submitted for:

- Royal College of Obstetricians and Gynaecologists Annual Academic Meeting - Royal College of Obstetricians and Gynaecologists, London - 9th – 10th Feb 2018
- The British Society for Immunology: Edinburgh Immunology Group Summer Symposium - University of Edinburgh - 24th - 26th April

Abstract:

Exploring the role of macrophage sub-populations in the aetiology of endometriosis

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Introduction: Endometriosis is a chronic inflammatory disorder, associated with debilitating pain and infertility, characterised by endometrial-like tissue (lesions) outside the uterus. The aetiology of endometriosis is poorly understood. Tissue-resident macrophages are a heterogeneous population of immune cells that fulfil tissue-specific functions. Macrophages are elevated in peritoneal fluid of women with endometriosis compared to women without disease, and endometrium tissue-resident macrophages (ETRM) are present in endometriosis lesions. We explored the role of ETRM, peritoneal and monocyte-derived macrophages (MDM) in endometriosis lesion development.

Methods: Using our validated in-house mouse model of endometriosis, we used dual immunofluorescence to investigate the proliferative capacity of ETRM (Ki67+, GFP+; ETRM labelled with GFP) and to identify large peritoneal macrophages (F4/80+, GATA6+) in endometriosis lesions. To assess the role of MDM, we used the same mouse model comparing CCR2 -/- (deficient in MDM) and wild-type mice, and assessed the resulting numbers of endometriosis lesions and quantified peritoneal macrophage populations by flow cytometry.

Results: Co-expression of GFP+ macrophages with the proliferation marker Ki67 in endometriosis lesions indicated their potential to self-renew. Only 4% of endometriosis lesion-resident macrophages were GATA6+ F4/80+large peritoneal macrophages.CCR2 -/- mice developed endometriosis lesions, and had more peritoneal Ly6C+ macrophages compared to sham CCR2 -/- mice (P=0.03).

Conclusions: ETRM may play a key role in endometriosis lesion biology and the presence of endometriosis induces recruitment of monocytes through a CCR2-independent mechanism. Targeting ETRM, or preventing the influx of ‘pro-disease’ macrophage populations into lesions, could be a novel approach for treating endometriosis.

Submitted for:

- A Cell for All Seasons: Macrophage Satellite Symposium - University of Edinburgh – Tuesday 1st May 2018

Abstract:

Monocyte-derived macrophages: evidence for a novel role in endometriosis

Chloe Hogg, Andrew W Horne, Jeffrey Pollard, Erin Greaves

MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, Scotland

Introduction: Endometriosis is a chronic inflammatory disorder, associated with debilitating pelvic pain and infertility and is characterised by the growth of endometrial-like tissue (lesions) outside the uterus. The aetiology of endometriosis is poorly understood however macrophages are important for growth, vascularization and innervation of lesions. Our research has shown that macrophages in lesions have different ontogenies, however the contribution of monocyte-derived macrophages to this population is unknown. We hypothesised that inhibition of recruited monocytes would reduce development of lesions.

Methods: Endometriosis was induced in WT(n=6) and CCR2 -/- (n=6) mice (deficient in monocyte-derived macrophages) using our in-house model. Peritoneal and lesion macrophage populations were assessed by flow cytometry and immunofluorescence.

Results: CCR2 -/- mice developed the same number of lesions as WT. Interestingly, CCR2 -/- mice with endometriosis had an increase ($p<0.05$) in monocytes in the peritoneal cavity compared to controls. In WT mice 17% (±SEM 6%) of total cells within lesions were monocyte-derived macrophages, 3% (±SEM1%) were monocytes and 8%(±SEM 2%) were mature macrophages. This was further confirmed by immunofluorescent staining.

Conclusions: The presence of lesions in the peritoneal cavity induces extravasation of monocytes independent of CCR2. Monocytes and monocyte-derived macrophages are a significant proportion of cells in endometriosis lesions.

Submitted for:

- The Society for the Study of Reproduction 2018 Annual Conference - New Orleans, Louisiana - 10th-13th July 2018

Abstract:

Exploring macrophage heterogeneity in endometriosis: novel evidence supporting a role for monocyte-derived macrophages in lesion development.

Chloe Hogg, Andrew W Horne, Jeffrey W Pollard, Erin Greaves

MRC Centre for Reproductive Health, University of Edinburgh, Edinburgh, United Kingdom

Endometriosis is a common chronic inflammatory disorder associated with debilitating chronic pelvic pain and infertility that affects ~176 million women worldwide. It is defined by the presence of endometrial-like tissue outside the uterus (endometriosis lesions), commonly on the pelvic peritoneum. The current management of women with endometriosis is unsatisfactory and there is an unmet clinical need for new medical treatments. Immune cells, called macrophages, play a role in the growth, vascularization and innervation of endometriosis lesions, and offer a potential therapeutic target. We have previously shown that lesion-resident macrophages are a heterogeneous population, consisting of tissue-resident endometrial macrophages, as well as macrophages originating either from the peritoneal cavity or derived from peripheral blood monocytes. The exact contribution of peritoneal and monocyte-derived macrophages is unknown. The objective of this study was to define the role of these macrophage populations in endometriosis using our in-house mouse model. To assess infiltration of tissue-resident large peritoneal macrophages (LPM) into lesions, we used dual immunofluorescence for LPM markers F4/80 and GATA6. 4% (±SEM 2%) of lesion-resident macrophages co-expressed these markers suggesting they were indeed LPM (n=10). To assess the incorporation of monocyte-derived macrophages into lesions, we interrogated mouse lesions by flow cytometry (n=6). 17% (±SEM 6%) of total cells within lesions were F4/80^{hi}, Ly6C+monocyte-derived macrophages, 3% (±SEM 1%) were F4/80-, Ly6C+ monocytes and 8%(±SEM 2%) were F4/80^{hi}, Ly6C^{-} mature macrophages. We validated our findings using dual immunofluorescence for F4/80 and Ly6C. Since monocyte-derived macrophages represented a significant number of cells in lesions we postulated that they might play an important role in lesion development. In order to investigate this, we used C-C chemokine receptor type 2 (CCR2) -/- mice, which are deficient for the chemokine (C-C motif) ligand 2 (CCL2) receptor. CCL2 is a key chemokine responsible for the extravasation of monocytes into the peritoneal cavity, therefore these mice are deficient in monocytes and monocyte-derived macrophages in this cavity. We induced endometriosis in both wild-type (n=6) and CCR2 -/- (n=6) mice and assessed the number of lesions that developed. There was no difference in the number of lesions recovered from CCR2 -/- mice compared to WT. CCR2 -/- mice with endometriosis had an increase (p=0.03) in monocytes in the peritoneal cavity compared to sham controls (n=8). This suggests that lesions produce a strong chemotactic signal that stimulates the extravasation of monocytes, which in this model is independent of CCR2. Our on-going work aims to characterise which chemokine is responsible for the extravasation of monocytes in our model and assess whether blocking this pathway could stop endometriosis lesions developing. In summary, we have shown that lesion-resident macrophages have different origins, and include endometrial, peritoneal and monocyte-derived macrophages. We have also demonstrated that lesions cause the extravasation of monocytes into the peritoneal cavity in a CCR2-independent manner. Thus, monocyte-derived macrophages may play an important role in endometriosis lesion development, and future therapies that target infiltration of monocytes may be of benefit in the treatment of women with endometriosis.

Submitted for:

- The Society for Reproductive Investigation 66th Annual Scientific Meeting - Paris, France – 12th-16th March 2019

Abstract:

Phenotypic characterisation of lesion-resident and peritoneal macrophages in a mouse model of endometriosis

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MRC Centre for Reproductive Health, University of Edinburgh, United Kingdom

Introduction: Endometriosis is a chronic inflammatory estrogen-dependent disorder defined by the presence of endometrial-like tissue outside the uterus (lesions), estimated to affect ~176 million women worldwide. Current treatment options are unsatisfactory and there is an unmet need for new medical therapies. Macrophages play a role in the growth, vascularization and innervation of lesions, and offer a potential therapeutic target. However, the phenotype of macrophages in endometriosis lesions is underexplored. We hypothesised that endometriosis macrophages possess a lesion-specific phenotype that is independent of ontogeny.
Methods: We induced endometriosis in WT mice using donor 'menses' endometrial tissue from Csf1r-EGFP mice (macrophages express GFP), allowing us to distinguish GFP+ endometrial macrophages in the resulting lesions. Lesions and peritoneal lavage were analysed by flow cytometry.
Results: 16% (SEM± 4%) of macrophages in lesions were GFP+ endometrial macrophages (N=6). These cells had a homogeneous phenotype (CX3CR1+ CCR2+ MHC II- CD86+). Of the GFP- host derived macrophages 28.4% (SEM± 3%) had the same phenotype as the endometrial macrophages. We also identified another four phenotypes of GFP- macrophages that were MHC11+ and CCR2-. Each population accounted for 10% of total macrophages. Macrophage phenotypes in lesions were consistent across animals. We found more CCR2+ macrophages in the peritoneal cavity of mice with endometriosis compared to naive ($p=0.005$) and sham animals ($p=0.01$) (N=6). The number of macrophages expressing CCR5, CD200, CD86, MHC II and CX3CR1 was similar in peritoneal macrophages from endometriosis mice compared to sham mice.
Conclusions: The phenotype of lesion-resident macrophages appears to be largely independent of origin and consistency across animals suggests that a predominant 'lesion-specific' macrophage signature exists. We suggest that, once incorporated into lesions, macrophages obtain a defined, pro-disease phenotype in response to local micro-environmental clues. Other macrophage phenotypes are present but the dynamics of these cells currently remains unknown. This data indicates that macrophages in lesions possess a predictable phenotypic signature and contributes to the aim of developing macrophage-targeted therapies for women with endometriosis.

Submitted for:

- The British Society for Immunology, Inflammation: from Initiation to Restoration Conference - University of Edinburgh - 24th - 26th April 2019

- The Society for the Study of Reproduction 52nd Annual Conference, Beyond Possible: Remarkable Transformation of Reproductive Biology - San Jose, California – 18th-21st July 2019

Abstract:

Macrophages in endometriosis exhibit phenotypic heterogeneity and have potential as therapeutic targets

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2. Centre for Inflammation Research, University of Edinburgh, Edinburgh, United Kingdom

Endometriosis is a chronic inflammatory disorder associated with pelvic pain and infertility that affects ~176 million reproductive age women worldwide. It is defined by the presence of endometrial-like tissue outside the uterus (lesions). Current management is unsatisfactory and there is an unmet need for new treatments. Macrophages play a key role in the growth, vascularization and innervation of endometriosis lesions, however our knowledge of macrophage origins, phenotype and heterogeneity in endometriosis is limited. We aimed to define the origin of lesion-resident macrophages and assess phenotypic heterogeneity using our unique mouse model. To assess infiltration of tissue-resident large peritoneal macrophages (LPM) into lesions, we adoptively transferred LPM (isolated from Csf1r-EGFP mice) into the peritoneal cavity of endometriosis mice. GFP+ cells were identified by immunohistochemistry in lesions after 2 weeks (n=4). To assess infiltration of $Ly6C^{hi}$ monocytes into lesions we performed Ly6C, F4/80 dual immunofluorescence. We identified both $Ly6C^{+}$ monocytes and $F4/80^{+}$ mature macrophages in lesions, which was also validated using flow cytometry (n=6). Next, we induced endometriosis in WT mice using donor endometrial tissue from Csf1r-EGFP mice; we identified that16% (SEM± 4%) of lesion macrophages were GFP+ endometrial-derived macrophages (n=6). Using single-cell RNA-seq, we performed unbiased transcriptional profiling of CD45+ cells in lesions (6006 cells), donor endometrial tissue (1306 cells) and peritoneal lavage cells from sham (5645 cells) and endometriosis mice (6720 cells). Clustering analysis identified 4 lesion-resident macrophage populations. Comparison of lesion-resident macrophage populations with macrophage clusters from endometrial tissue and the peritoneum confirmed that lesions contained macrophages from different origins. In summary, we demonstrate, for the first time, that macrophages in endometriosis lesions are heterogeneous in both ontogeny and transcriptional profile. We believe that this observed heterogeneity could be translated into clinical applications, such as targeted therapy for endometriosis-associated pain.

Posters from Thesis

Peritoneal macrophage dynamics in a mouse model of endometriosis

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Background:

Endometriosis is a chronic inflammatory disorder characterised by the presence of endometrial-like tissue (lesions) outside the uterus, most commonly in the peritoneal cavity (1). Macrophages are critical for angiogenesis and cell proliferation in endometriosis lesions (2), however little is known about the origins of these cells. In a mouse model of endometriosis we have previously shown that donor endometrial macrophages exist in endometriosis lesions but a larger proportion of lesion-resident macrophages are host derived (3). One possible origin of host-derived lesion-resident macrophages could be peritoneal macrophages. In the peritoneal cavity macrophages exist as two populations; large peritoneal macrophages (LPM), usually high in number and associated with immune surveillance and homeostasis, and small peritoneal macrophages (SPM), usually low in number and are associated with inflammation (4).

Hypothesis

Mice with endometriosis exhibit alterations in peritoneal myeloid cell number and peritoneal macrophages become incorporated into endometriosis lesions.

Methods:

Using our established mouse model of endometriosis (3) [naïve (n=8) and sham+E2 (n=6) controls for comparison], peritoneal lavage using 7ml DMEM was performed and populations of large and small peritoneal macrophages as well as monocytes were enumerated by flow cytometry (n=10). Dual immunofluorescence was performed on mouse lesions using large peritoneal macrophage markers GATA6 and F4/80. Statistical analysis was performed using a one-way ANOVA and Newman Keuls post-hoc test with a significance level of p<0.05.

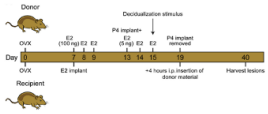

Figure 1. The Edinburgh mouse model of endometriosis. Donor mice are ovariectomised and receive hormonal treatments which mimic the human menstrual cycle, producing donor mouse 'menstrual' material for injection into the peritoneal cavity of recipient mice, which develop human-like endometriosis lesions.

Results:

1. *Mice with endometriosis do not have significantly altered numbers of large or small peritoneal macrophages*

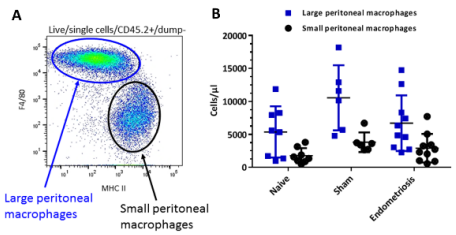

Figure 2. Large and small peritoneal macrophages in mice with endometriosis. Large and small peritoneal macrophages were distinguished based on expression of F4/80 (LPM) and MHC II (SPM) (**A**). There was no significant difference in large peritoneal macrophage numbers between any of the groups (P=0.073). Mean LPM numbers were 7299 cells/µl in mice with endometriosis which was higher than naïve mice (5382 cells/µl) but lower than sham mice (10554 cells/µl). A similar, non-significant trend was seen in the small peritoneal macrophage population with no significant difference between any of the groups (P=0.087) (**B**).

2. *Mice with endometriosis do not have significantly altered numbers of small peritoneal macrophage sub-populations*

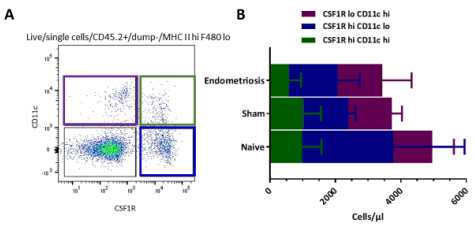

Figure 3. Sub-populations of small peritoneal macrophages in mice with endometriosis. The F4/80^{lo} MHC II^{hi} (SPM) population could be separated into CSF1R^{hi} CD11c^{hi}, CSF1R^{hi} CD11c^{lo}, and CSF1R^{lo} CD11c^{hi} (dendritic cells) (**A**). There was no significant difference between groups in any of the SPM sub-populations (**B**).

3. *A trend for increased monocyte number in the peritoneal cavity of mice with endometriosis*

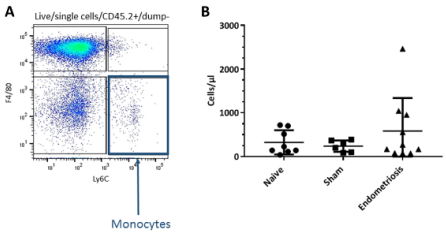

Figure 4. Monocytes in the peritoneal cavity of mice with endometriosis. Monocytes were gated as Ly6C^{hi} F4/80^{lo} cells (**A**). Mean cells/µl in each group were 327 (±SEM 98) in the naïve animals, 241 (±SEM 53) in the sham group, and 705 (±SEM 230) in the endometriosis group, although this was not statistically significant (P=0.917) (**B**).

4. *Large peritoneal macrophages represent 4% of lesion-resident macrophages*

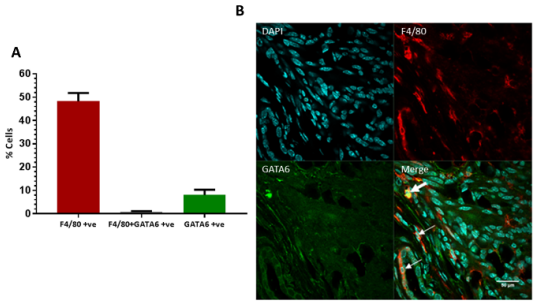

Figure 5. Large peritoneal macrophages in the endometriosis lesions. 48% (±SEM 4%) of total DAPI^{+} cells in lesions expressed F4/80, and 4% (±SEM 2%) of these were F4/80^{+} GATA6^{+} large peritoneal macrophages (n=10 lesions from n=7 animals). A population of GATA6^{+} cells could also be detected which lacked F4/80 expression, and these contributed to 8% (±SEM 2%) of the overall cell population (**A**). F4/80^{+} cells could be seen both throughout lesions as well as clustered around glands and blood vessels and in some lesions accounted for up to 89% of total cells (**B**).

Conclusions:

1. **Mice with endometriosis do not have significantly different numbers of small or large peritoneal macrophages**
2. **Mice with endometriosis have higher numbers of peritoneal monocytes**
3. **Large peritoneal macrophages represent 4% of lesion-resident macrophages**

References

1. Linda C, Giudice M: Endometriosis. N Engl J Med 2010, 362: 2389-2398
2. Bacci M *et al*: Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. Am J Pathol 2009, 175: 547-556
3. Greaves E *et al*: A Novel Mouse Model of Endometriosis Mimics Human Phenotype and Reveals Insights into the Inflammatory Contribution of Shed Endometrium. Am J Pathol 2014, 184: 1930-1939
4. Bain C *et al*: Long-lived self-renewing bone marrow-derived macrophages displace embryo-derived cells to inhabit adult serous cavities. Nat Com 2016, 7: 11852

Exploring the role of macrophage sub-populations in the aetiology of endometriosis

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Introduction

- There is an unmet clinical need for new treatments for endometriosis[1].
- Immune cells called macrophages play a key role in the growth, vascularisation and innervation of endometriosis lesions[2,3].
- The aim of this study was to explore the role of peritoneal and monocyte-derived macrophages in endometriosis lesion development.

Methods

Endometriosis was induced in mice using our established mouse model4 (Fig.1). Dual immunofluorescence for peritoneal macrophage markers GATA6 + F4/80 and monocyte/macrophage markers Ly6C + F4/80 was performed on lesions. Flow cytometry was used to assess infiltration of monocytes (F4/80$^-$ Ly6C$^+$), monocyte-derived macrophages (F4/80$^+$ Ly6C$^+$) and mature macrophages (F4/80$^+$ Ly6C$^-$) in lesions. Endometriosis was induced in CCR2 -/- mice (deficient in monocyte-derived macrophages) alongside wild type (WT) mice and lesions assessed.

Results

- 4% of macrophages in lesions are peritoneal macrophages (Fig.2)
- Monocytes infiltrate endometriosis lesions (Fig.3)
- Mice deficient in monocyte-derived macrophages develop the same number of lesions as wild type mice (Fig.4).

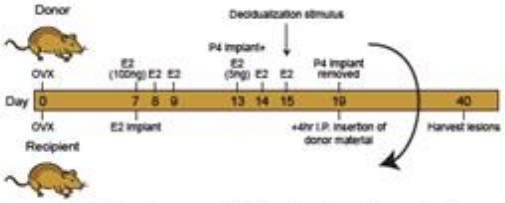

Figure 1. The Edinburgh mouse model of endometriosis. We produce donor mouse 'menstrual' tissue which is injected into the peritoneal cavity of recipient mice, which develop human-like endometriosis lesions[4].

References

1. Linda et al, Endometriosis: N Engl J Med 2010, 362: 2389-2398
2. Bacci et al, Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease: Am J Pathol 2009, 175: 547-556
3. Greaves et al, Estradiol Is a Critical Mediator of Macrophage-Nerve Cross Talk in Peritoneal Endometriosis: Am J Pathol 2015, 185: 2286-2297
4. Greaves et al, A Novel Mouse Model of Endometriosis Mimics Human Phenotype and Reveals Insights into the Inflammatory Contribution of Shed Endometrium: Am J Pathol 2014, 184: 1930-1939

4% of macrophages in lesions are peritoneal macrophages

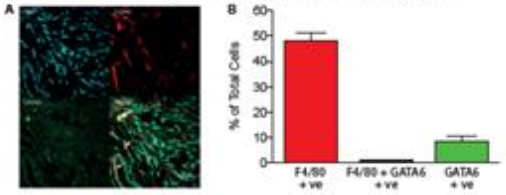

Figure 2. Peritoneal macrophages in endometriosis lesions. (A/B) 48% (±SEM 4%) of total DAPI$^+$ cells in lesions expressed F4/80, and 4% (±SEM 2%) of these were F4/80$^+$ GATA6$^+$ large peritoneal macrophages.

Monocytes infiltrate endometriosis lesions

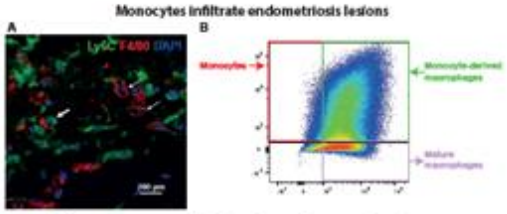

Figure 3. Monocytes, monocyte-derived macrophages and mature macrophages in mouse endometriosis lesions. (A) Monocytes (Ly6C$^+$) and macrophages (F4/80$^+$) were in lesions. **(B)** 17% (±SEM 6%) of total cells within the lesion were monocyte-derived macrophages (Ly6C$^+$ F4/80$^+$), 3%(±SEM1%) were monocytes (Ly6C$^+$ F4/80$^-$) and 8%(±SEM 2%) were mature macrophages (Ly6C$^-$ F4/80$^+$).

Mice deficient in monocyte-derived macrophages develop the same number of lesions as wild type mice

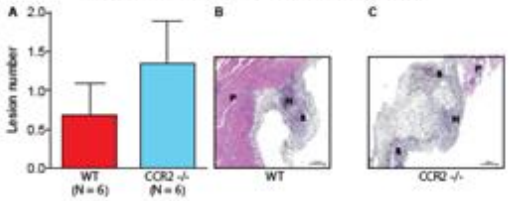

Figure 4. Lesion number and histology in wild type and CCR2 -/- mice with induced endometriosis. (A) The same number of lesions were recovered from WT and CCR2-/- **(B)** Haematoxylin and eosin staining showed expected lesion morphology in WT and CCR2 -/- mice. P, peritoneum; S, stromal cells; H, hemosiderin.

Conclusions

- Monocyte derived macrophages may not be important for endometriosis lesion development.
- Peritoneal macrophages are a minor population in endometriosis lesions.
- Endometriosis could be treated by targeting endometriosis-specific macrophage populations.

Exploring macrophage heterogeneity in endometriosis: novel evidence supporting a role for monocyte-derived macrophages in lesion development

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Introduction

- There is an unmet clinical need for new treatments for endometriosis[1].
- Immune cells called macrophages play a key role in the growth, vascularisation and innervation of endometriosis lesions and are a potential therapeutic target[2,3].
- CCL2 is a chemokine required for the extravasation of macrophage precursor cells (monocytes) from blood vessels into tissues. It also mediates macrophage chemotaxis[4].
- We have previously shown that expression of CCL2 is up-regulated in endometriosis lesions derived from our mouse model[5].

Aim

The aim of this study was to explore the role of monocyte-derived macrophages and the CCL2-CCR2 chemokine pathway in endometriosis lesion development.

Methods

Endometriosis was induced in mice using our established mouse model[5] (Fig.1). Flow cytometry was used to assess infiltration of monocytes (F4/80- Ly6C+), and the presence of monocyte-derived macrophages (F4/80+ Ly6C+) and mature macrophages (F4/80+ Ly6C-) in lesions. Peritoneal lavage cells were also analysed by flow cytometry in order to assess expression of the monocyte/macrophage chemokine receptor CCR2. Endometriosis was induced in CCR2 -/- mice (deficient in monocyte-derived macrophages in the peritoneal cavity) alongside wild type (WT) mice and lesions number/histology assessed and peritoneal monocyte/macrophage populations enumerated.

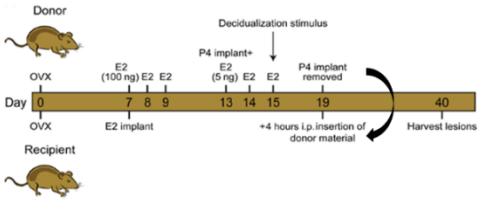

Figure 1. The Edinburgh mouse model of endometriosis. Donor mice are ovariectomized and receive hormonal treatments which mimic the human menstrual cycle, producing donor mouse 'menstrual' material for injection into the peritoneal cavity of recipient mice, which develop human-like endometriosis lesions[5].

Results

- Monocyte-derived macrophages infiltrate endometriosis lesions (Fig.1).
- Mice with endometriosis have elevated numbers of CCR2+ macrophages in the peritoneal cavity (Fig.2).
- CCR2 -/- mice develop the same number of lesions as WT, and have an un-expected influx of Ly6C^{hi} monocytes into the peritoneal cavity (Fig.3).

Monocyte-derived macrophages infiltrate endometriosis lesions

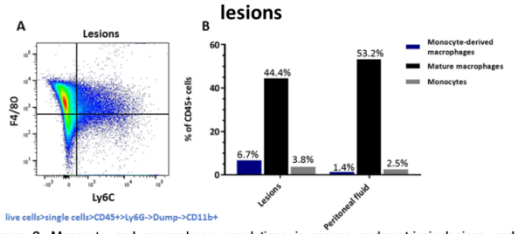

Figure 2. Monocyte and macrophage populations in mouse endometriosis lesions and peritoneal lavage fluid. (A) Representative flow plot **(B)** Within lesions, 6.7% of CD45+ cells were monocyte-derived macrophages, 44.4% were mature macrophages and 3.8% were monocytes. In peritoneal lavage fluid, there was a lower percentage (1.4%) of monocyte-derived macrophages in the CD45+ population, a higher percentage (53.2%) of mature macrophages and a lower percentage (2.5%) of monocytes.

Mice with endometriosis have more peritoneal CCR2+ macrophages

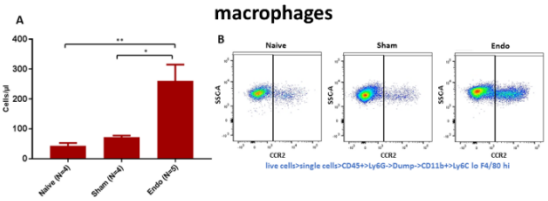

Figure 3. CCR2+ macrophages in the peritoneal cavity of naïve, sham and endometriosis mice. (A) Mice with endometriosis had significantly more CCR2+ macrophages in the peritoneal cavity compared to naïve (p=0.005) and sham mice (p=0.01). **(B)** Representative flow plots.

CCR2 -/- mice develop endometriosis and have an influx of Ly6C^{hi} monocytes into the peritoneal cavity

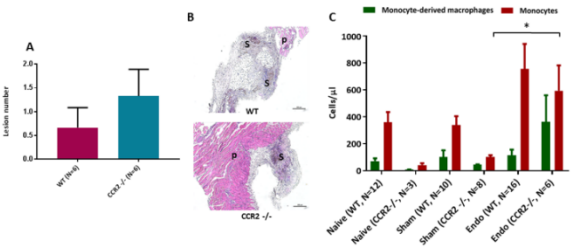

Figure 4. Lesion number, histology and peritoneal macrophage populations in wild type and CCR2 -/- mice with induced endometriosis. (A) The same number of lesions were recovered from WT and CCR2 -/- mice **(B)** Haematoxylin and eosin staining showed expected lesion morphology in WT and CCR2 -/- mice. P, peritoneum; S, stromal cells. **(C)** Flow cytometry analysis of peritoneal monocyte and macrophage populations revealed that CCR2 -/- mice with endometriosis had an increase in Ly6C^{hi} monocytes in the peritoneal cavity compared to sham (p=0.03).

Conclusions

1. Monocyte-derived macrophages can be found in mouse endometriosis lesions, suggesting they could play a role in lesion development.
2. The presence of endometriosis lesions causes the extravasation of Ly6C^{hi} monocytes into the peritoneal cavity, which in CCR2-/- mice is independent of CCR2.
3. Targeting the influx of Ly6C^{hi} monocytes may inhibit the growth of lesions and be of therapeutic benefit.

Future directions

We plan to identify which chemokine is responsible for the extravasation of Ly6C^{hi} monocytes in CCR2-/- mice with endometriosis and decipher the effect of blocking this on growth of lesions.

References

1. Linda C, Giudice M: Endometriosis. N Engl J Med 2010, 362: 2389-2398
2. Bacci M *et al*: Macrophages are alternatively activated in patients with endometriosis and required for growth and vascularization of lesions in a mouse model of disease. Am J Pathol 2009, 175: 547-556
3. Greaves *et al*, Estradiol Is a Critical Mediator of Macrophage-Nerve Cross Talk in Peritoneal Endometriosis: Am J Pathol 2015, 185: 2286-2297
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5. Greaves E *et al*: A Novel Mouse Model of Endometriosis Mimics Human Phenotype and Reveals Insights into the Inflammatory Contribution of Shed Endometrium. Am J Pathol 2014, 184: 1930-1939

Phenotypic Characterisation of Lesion-Resident and Peritoneal Macrophages in a Mouse Model of Endometriosis

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Background:

There is an unmet clinical need for new treatments for endometriosis[1]. Macrophages play a key role in the growth, vascularisation and innervation of endometriosis lesions and are a potential therapeutic target[2,3]. The phenotype of lesion-resident macrophages is largely under-explored. Few markers have been analysed which suggest a 'wound healing' phenotype, however the complexity of macrophage phenotype is yet to be revealed. There have been no transcriptional studies on endometriosis lesion-resident macrophages.

Hypothesis:

Endometriosis lesion-resident macrophages are derived from endometrial, peritoneal and monocyte-derived macrophages and exhibit phenotypic heterogeneity.

Methods:

Endometriosis was induced in mice using our established mouse model[4] (Fig.1). We used Csf1r-EGFP (macrophages express GFP)[5] donor mice to visualize GFP+ endometrial-derived macrophages in lesions. Lesions, peritoneal lavage fluid and decidualized endometrial tissue were analysed by flow cytometry for macrophage activation markers which are indicative of either a pro-inflammatory (CCR2, MHC II, CD86) or wound-healing phenotype (CX3CR1). To determine large peritoneal macrophage (LpM) infiltration into lesions we adoptively transferred 4 x 10^6 GFP+ LpM into the peritoneal cavity of endometriosis mice. GFP+ F4/80^{hi} MHC II^{lo} LpM were isolated from Csf1r-EGFP mice by FACS sorting. 2 weeks later lesions were dissected, fixed in NBF and stained for GFP. We stained endometriosis lesions for Ly6C and F4/80 to assess infiltration of monocytes into the tissue. For single cell RNA sequencing (scRNA-Seq), endometriosis lesions, donor endometrial tissue and peritoneal cells from sham and endometriosis mice were sorted by FACS to isolate CD45+ cells. The samples were taken through established pipelines for single cell discovery and NovaSeq Illumina sequencing by synthesis performed.

The Edinburgh mouse model of endometriosis mimics retrograde menstruation

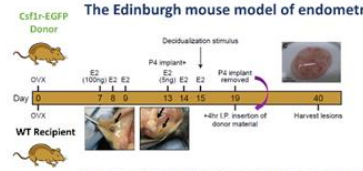

Figure 1. The Edinburgh mouse model of endometriosis. Donor mice are ovariectomized and receive hormonal treatments which mimic the human menstrual cycle, producing mouse 'menstrual' endometrium for injection into the peritoneal cavity of recipient mice, which develop endometriosis lesions that phenocopy those seen in women[4].

Heterogeneity of endometrial macrophages is lost upon incorporation into lesions

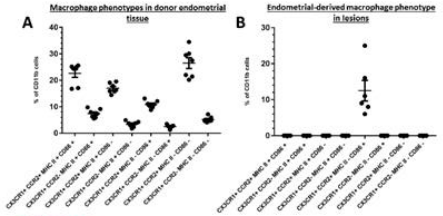

Figure 2. The phenotype of endometrial macrophages in donor endometrial tissue and endometriosis lesions. **(A)** Donor 'menses' endometrial tissue was analysed by flow cytometry for 4 macrophage activation markers (n=7 mice). We were able to distinguish 8 phenotypically distinct populations. **(B)** Using flow cytometry, GFP+ endometrial derived macrophages in lesions formed a single population that was CX3CR1+ CCR2+ CD86- MHC II+ (n=6 mice). 16% (±SEM 4%) of lesion macrophages were endometrial-derived.

References:
1: Giudice, L.C., Kao L.C. The Lancet. 2004; 364, 1789-1799
2: Tokushige, N., et al. Human Reproduction. 2006; 21(11), 3001-3007
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Large peritoneal macrophages and Ly6C^{hi} monocytes infiltrate lesions and exhibit phenotypic heterogeneity

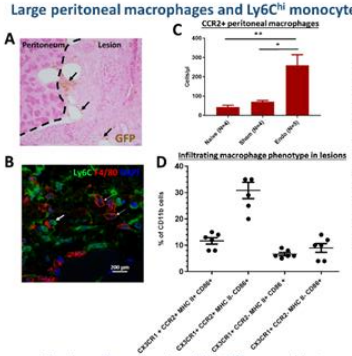

Figure 3. Large peritoneal macrophage and Ly6C^{hi} monocyte incorporation into lesions and phenotypic characterisation of infiltrating macrophages. **(A)** We detected GFP+ LpM in lesions 2 weeks after adoptive transfer of cells (n=4 mice). **(B)** Ly6C^{hi} monocytes and F4/80^{hi} mature macrophages were identified in endometriosis lesions. **(C)** Mice with endometriosis had more CCR2+ peritoneal macrophages compared to naïve (p=0.005) and sham (p=0.01). **(D)** GFP- infiltrating macrophages in lesions formed four phenotypically distinct populations based on markers assessed. 31% (±SEM 3%) of these shared the same phenotype as endometrial-derived lesion macrophages (n=6 mice).

Single cell sequencing identifies novel heterogeneity in endometriosis lesion resident macrophages

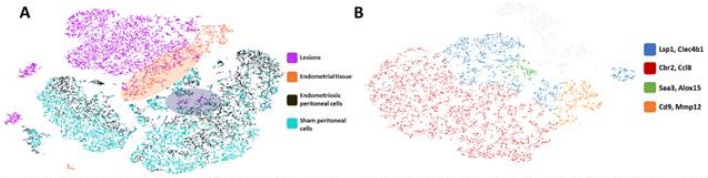

Figure 4. t-SNE plots showing graph-based clustering of mouse endometriosis lesions, donor endometrial tissue and peritoneal cells from endometriosis and sham mice. **(A)** t-SNE plot of all samples. A proportion of lesion-resident macrophages clustered with both endometrial (orange circle) and peritoneal macrophages (black circle), further validating our evidence supporting different origins. **(B)** t-SNE plot of endometriosis lesions. From the lesion-resident CD45+ cells we were able to distinguish 4 sub-populations of Agre1$^+$ Csf1r$^+$ Itgam$^+$ macrophages. Key shows a selection of upregulated genes which define each cluster.

Conclusions:

- **Endometriosis lesion-resident macrophages are derived from endometrial, peritoneal and monocyte-derived macrophages and infiltrating macrophages exhibit phenotypic heterogeneity.**
- **In scRNA-Seq analysis, lesion macrophages cluster with both endometrial and peritoneal macrophages, confirming our ontogeny studies.**
- **Lesion macrophages form 4 sub-populations in scRNA-Seq identifying novel transcriptomic heterogeneity in this population which has thus far been unidentified, opening up new possibilities for therapeutic intervention of specific, pathogenic macrophage populations.**

Abbreviations

rASRM	Revised American Society for Reproductive Medicine Score
EFI	Endometriosis Fertility Index
GnRH	Gonadotrophin-releasing hormone
GWAS	Genome-wide association study
17β-HSD	17β-hydroxysteriod dehydrogenase
FOXN1	Forkhead box protein N1
Prkdc	DNA-dependent protein kinase
EMP	Erythro-myeloid progenitor
CCL2	Chemokine (C-C motif) ligand 2
CCR2	C-C chemokine receptor type 2
GM-CSF	Granulocyte-macrophage colony-stimulating factor
IFN-γ	Interferon γ
M-CSF	Macrophage colony-stimulating factor
IL	Interleukin
LPM	Large peritoneal macrophage
SPM	Small peritoneal macrophage
Gata6	GATA-binding factor 6
IRF4	Interferon regulatory factor 4
RELMα	Resistin-like molecule α
CD206	Mannose receptor
CRIg	Complement receptor of the immunoglobulin superfamily
Tim4	T-cell immunoglobulin and mucin domain containing 4
CSF1R	Colony stimulating factor 1
DAMP	Damage-associated molecular pattern
ATP	Adenosine triphosphate
CCL5	Chemokine (C-C motif) ligand 5
CCL3	Chemokine (C-C motif) ligand 3
TNF- α	Tumour necrosis factor-α
IL-1β	Interleukin-1β
TAM	Tumour associated macrophage

Tie2	Angiopoietin receptor-2
CX3CR1	CX3C chemokine receptor 1
MMP	Matrix Metallopeptidase
VEGF	Vascular endothelial growth factor
ER	Estrogen receptor
CD	Cluster of differentiation
IGF-1	Insulin-like growth factor 1
iNOS	Nitric oxide synthase
MHC II	Major histocompatibility complex II
rtTA	Reverse tetracycline-controlled transactivator
GFP	Green fluorescent protein
EGFP	Enhanced green fluorescent protein
DAPI	4′,6-diamidino-2-phenylindole
PBS	Phosphate buffered saline
DMEM	Dulbecco's modified eagle medium
EV	Estradiol valerate
NaOH	Sodium hydroxide
EDTA	Ethylenediaminetetraacetic acid
Tris-Hcl	Tris-hydrochloric acid
dH_20	Distilled water
PCR	Polymerase chain reaction
RBC	Red blood cell
NBF	Neutral buffered formaldehyde
FACS	Fluorescence-activated cell sorting
DAB	3,3-diaminobezidine
FBS	Foetal bovine serum
CAG-Luc	FVB-Tg(CAG-luc,-GFP)L2G85Chco/J mice
GEM	Gel Bead-In Emulsion
UMI	Unique molecular identifier
Endo	Endometriosis
PF	Peritoneal lavage fluid
cKO	Conditional knock out

KO	Knock out
WT	Wild-type
MacGreen	Csf1r-EGFP transgenic mice
tSNE	T-distributed stochastic neighbour embedding
bp	Base pair

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Chapter 1 - Introduction

1.1 Endometriosis

1.2 The clinical problem

Endometriosis is defined by the presence of endometrial-like tissue outside the uterus, typically on the peritoneal wall of the pelvic cavity. The condition is estimated to affect 6-10% of women of reproductive age (Giudice, 2010) and up to 50% of infertile women (Meuleman*et al.*, 2009). Endometriosis is associated with debilitating chronic pelvic pain, infertility, dyspareunia (painful intercourse), dysmenorrhea (painful menstruation), dysuria (painful urination) and dyschezia (painful bowel movements). These symptoms can effect mental, physical and social wellbeing and negatively impact on quality of life (Dunselman *et al.*, 2014). The prevalence of endometriosis in women with chronic pelvic pain is 71-87% (Hansen, Chalpe and Eyster, 2010). Women with endometriosis may experience cyclic and/or non-cyclic pain which can be referred to other areas of the body for example the legs (Walch *et al.*, 2014). Endometriosis is also associated with reduced pregnancy rates and this is partly attributed to low oocyte number and quality as well as defective implantation in the uterus (Simón *et al.*, 1994; Garrido *et al.*, 2002; Xu *et al.*, 2015). Poor pregnancy outcomes are also associated with the disease, including preterm labour, pre-eclampsia, miscarriage and intrauterine growth restriction (Saraswat *et al.*, 2017). Endometriosis has a significant socioeconomic impact, costing the UK an estimated £8.5 billion pounds each year, with societal cost being mostly attributed to loss of productivity (Simoens *et al.*, 2012; Nnoaham *et al.*, 2013). Diagnosis from onset of symptoms can take an average of 6.7 years. Generally, a diagnosis of endometriosis is achieved by laparoscopic evaluation of the pelvis however imaging techniques such as transvaginal sonography and magnetic resonance imaging may be utilised to diagnose deep infiltrating lesions or endometriomas (Bazot and Daraï, 2017; Bedaiwy *et al.*, 2017; Agarwal *et al.*, 2019).

Endometriosis lesions are defined as ectopic endometrial-like tissue containing glands and stroma, however recent re-evaluation of disease definition suggests that fibrosis and smooth muscle cells are more consistent characteristics of lesions (Vigano *et al.*, 2018). Lesions are also infiltrated with blood vessels and nerves, and are most commonly observed on the ovaries, pelvic peritoneum and rectovaginal

septum (Capobianco *et al.*, 2011; Burney and Giudice, 2012; Greaves *et al.*, 2015). Endometriosis is a heterogeneous disease which consists of three sub-types: superficial peritoneal, deep (infiltrating) and ovarian ("endometriomas"), where more than one sub-type can exist in the same patient and superficial peritoneal endometriosis is the most common form of disease (Mahmood and Templeton, 1991; Johnson *et al.*, 2017). Disease classification currently takes into account lesion size, location, extent of lesion infiltration into tissue and the presence of adhesions and is classed from stage I (minimal) to stage IV (severe) (Canis *et al.*, 1997). Whilst this system is useful for assessing extent of disease within the peritoneal cavity, classification of disease does not predict symptom severity, pregnancy success or relapse after surgery (Vercellini *et al.*, 2006; Ek *et al.*, 2015). Other classification systems have attempted to predict symptomatic factors such as the endometriosis fertility index (EFI) (Adamson and Pasta, 2010) and Enzian classification for deep endometriosis (Haas *et al.*, 2013). A consensus study by the World Endometriosis Society on the classification of endometriosis concluded that, until a better classification system is developed, patients would benefit from using multiple classification systems dependent on their concerns and needs (r-ASRM, EFI and Enzian classifications) (Johnson *et al.*, 2017). The heterogeneity of endometriosis and lack of correlation between disease phenotype and symptoms makes the development of an appropriate classification system challenging.

Current treatments for endometriosis aim to alleviate endometriosis-associated pain and/or to treat infertility associated with the disease and include surgical and medical management (Giudice, 2010; Johnson and Hummelshoj, 2013). Ovarian suppression limits activity and growth of lesions, leading to reduced pain symptoms. Common methods of ovarian suppression include oral contraceptives and gonadotrophin-releasing hormone (GnRH) agonists (Al Kadri *et al.*, 2009). Whilst ovarian suppression may alleviate pain symptoms, treatment is also contraceptive and therefore inappropriate for women wanting to conceive. Additionally, GnRH agonists are associated with side effects such as memory loss, insomnia and hot flushes in a recent study of endometriosis patients with long term use (Gallagher *et al.*, 2018). Treatments can also include non-steroidal anti-inflammatory drugs such as ibuprofen (Brown *et al.*, 2017), however long-term pain management for women

with endometriosis often encompasses a combination of treatments. As well as medical therapy, laparoscopic surgery to remove lesions can provide symptom relief in some patients, however up to 50% of women experience a relapse of symptoms within two years after surgery (Deura and Harada, 2014; Bedaiwy *et al.*, 2017). Infertility treatments are an option for women with endometriosis wishing to conceive. These treatments include assisted reproductive technologies such as *in vitro* fertilisation and controlled ovarian hyperstimulation, as well as laparoscopic excision of lesions (Lessey, 2000; Devroey, 2003; Vlahos, Economopoulos and Fotiou, 2010; Deura and Harada, 2014). Current treatment options lack significant clinically proven benefit and aim at alleviating symptoms, rather than treating disease (Dunselman *et al.*, 2014). Consequently, there is a compelling clinical need for new non-hormonal treatments that have fewer side effects and effectively treat endometriosis over a life course, without the need for repeated surgeries or suppression of fertility.

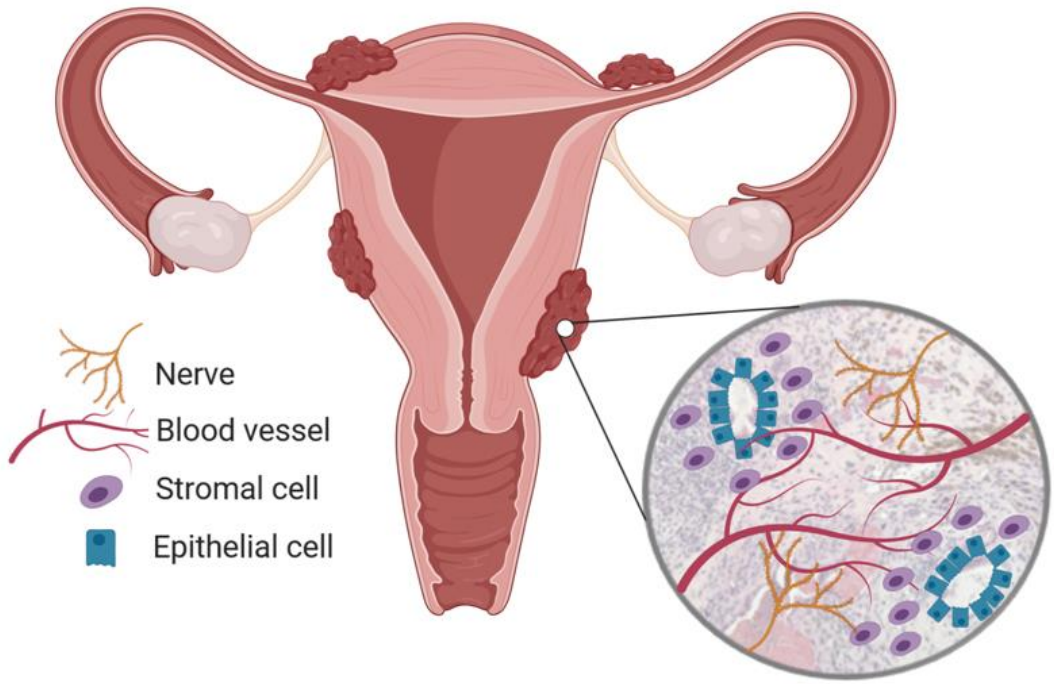


Figure 1.1. Endometriosis is a chronic inflammatory disorder. *Endometriosis is characterised by the presence of endometrial-like tissue found outside the uterus, most commonly in the peritoneal cavity. Endometriosis lesions are characterised by the presence of endometrial stromal cells and glands and are vascularised and innervated by nerves.*

1.1.2 Aetiology and natural history

Understanding of endometriosis aetiology is currently limited and a number of theories exist which attempt to explain disease pathogenesis. It is being increasingly recognised that different sub-types of endometriosis may arise from differential causal factors, however evidence for these theories still remains elusive (Burney and Giudice, 2012; Gordts, Koninckx and Brosens, 2017). A summary of current key theories of endometriosis aetiology is provided below.

1.1.2.1 Sampson's theory

The most widely accepted theory was postulated in 1927 by John Sampson, who observed that during menstruation, menses material can reflux back up the fallopian tubes and into the pelvic cavity, a physiological process known as 'retrograde menstruation'. Although this process is thought to occur in ~90% of women, only in some women does refluxed endometrial tissue adhere to the visceral or parietal peritoneum, forming lesions (Sampson, 1927). Women with endometriosis are thought to have higher volumes of refluxed menstrual blood, which supports this theory (Halme *et al.*, 1984). Women with congenital outflow obstruction have higher rates of endometriosis, as well as women with cervical stenosis or a uterine septum, where menstrual outflow is partially compromised (Sanfilippo *et al.*, 1986; Nawroth *et al.*, 2006). In a non-human primate model, obstruction of menstrual outflow results in endometriosis lesions in the peritoneal cavity (D'Hooghe *et al.*, 1994). The location of endometriosis lesions in the peritoneal cavity also supports the retrograde menstruation theory, with lesions frequently forming in the left hemisphere of the pelvis, attributed to decreased fluid movement in this area due to the placement of the sigmoid colon (Al-Fozan and Tulandi, 2003). Thus, refluxed endometrial tissue may remain in the left hemisphere of the pelvis for longer periods of time allowing implantation of endometrial deposits. This was demonstrated for both peritoneal lesions and endometrioma, with endometrioma being more common on the left ovary (Al-Fozan and Tulandi, 2003). However, whilst this hypothesis explains the presence of endometrial tissue in the peritoneal cavity, it does not account for the fact that only some women develop endometriosis when we consider retrograde menstruation

as a common physiological event (Sampson, 1927). Thus, other factors must be involved in endometriosis aetiology.

1.1.2.2 Neonatal uterine bleeding

Another theory based on the dissemination of cells from the uterus into the peritoneal cavity is the theory of neonatal retrograde reflux of endometrial stem/progenitor cells. This theory is based on the fact that 5-10% of neonates experience uterine bleeding, and thus retrograde bleeding is also possible (Brosens and Benagiano, 2013). During retrograde neonatal bleeding, stem/progenitor cells could implant into the peritoneal wall where they may remain dormant until sexual maturity is reached, where elevated estrogen levels could then cause the proliferation and growth of already seeded endometrial cells. Gordts *et al* hypothesised that superficial peritoneal disease could form as a result of ‘activation' of endometrial cells during puberty, and deep infiltrating lesions are a delayed stage of disease resulting from gradual growth of previously superficial lesions. They also suggest that the progression from superficial to deep infiltrating disease could be regulated by genetic/epigenetic factors (Gordts, Koninckx and Brosens, 2017). Whilst this theory represents a plausible mechanism of lesion formation, current evidence is lacking and proof that endometrial stem/progenitor cells are present in the peritoneal tissue of pre-pubescent girls is absent.

1.1.2.3 Coelomic metaplasia

Whilst a number of hypotheses are based on the dissemination of endometrial cells into the peritoneal cavity via retrograde menstruation, it is also thought that some lesions, for example endometriomas, arise from transformation of non-endometrial cells. Coelomic metaplasia is a hypothetical physiological event whereby endometriosis lesions arise as the result of metaplastic differentiation of the coelomic epithelium into endometrial cells (Konrad *et al.*, 2019). The coelomic metaplasia theory is supported by the fact that endometriosis lesions can be found in women with hysterectomy, meaning retrograde menstruation is redundant and lesions must be formed by another mechanism (Soliman *et al.*, 2017). The formation of endometriosis lesions in sites outside the peritoneal cavity also supports this theory,

where lesions have been found on the pericardium and the pleura (Jablonski *et al.*, 2009; Rousset-Jablonski *et al.*, 2011). Endometriosis lesions have also rarely been identified in men (Martin and Hauck, 1985). Whilst this evidence is supportive of the coelomic metaplasia theory, the mechanisms by which non-endometrial cells could be stimulated to differentiate into endometrial-like cells are unknown. This theory also does not explain why endometriosis is usually limited to the peritoneal cavity, and that incidence does not increase with age, as with other metaplastic diseases (Konrad *et al.*, 2019).

1.1.2.4 Heritability

It has long been appreciated that endometriosis has a heritable aspect due to high familial incidence of the disease (Simpson *et al.*, 1980; Treloar *et al.*, 1999; Nouri *et al.*, 2010). Genome-wide association studies (GWAS) employ a hypothesis-free approach to identify genetic variants in diseased and healthy populations. A meta-analysis of eight GWAS studies elucidated six loci associated with endometriosis (Rahmioglu *et al.*, 2014). Genes implicated in disease included those involved in the regulation of epithelial cells and hormone metabolism, specifically genes involved in regulating hormone responses in tissues (Nyholt *et al.*, 2012; Sapkota *et al.*, 2017). Functional studies into the roles of genes associated with incidence of endometriosis however are required to infer as to the downstream biological pathways they might affect (Rahmioglu *et al.*, 2014). Thus, genetic predisposition to endometriosis is likely to contribute to disease aetiology, however current understanding of the functional role of genes implicated in disease is lacking.

1.1.2.5 Immune cell dysregulation

Ectopic endometrial cells elicit an immune response in the peritoneal cavity and are exposed to a variety of different cytokines, chemokines and hormones as a result of this. Organs in the peritoneal cavity are bathed in peritoneal fluid, a fluid formed from ovarian exudate (Koninckx *et al.*, 1980) and plasma transudate (Oral, Olive and Arici, 1996). Alterations in immune cell populations have been observed in the peritoneal fluid of women with endometriosis; specifically, women with

endometriosis have more peritoneal macrophages (Haney, Muscato and Weinberg, 1981), neutrophils and dendritic cells (Tariverdian *et al.*, 2009).

Dysregulation of immune cell function in the peritoneal cavity have been reported in women with endometriosis. For example, a decrease in NK cell cytotoxicty has been observed in peritoneal NK cell populations of women with endometriosis (Oosterlynck *et al.*, 1991; Jeung, Cheon and Kim, 2016), and disease severity is positively correlated with NK cell cytotoxic killing capacity (Ho *et al.*, 1995). Dysregulation of peritoneal macrophage phagocytic capacity is also implicated in disease (Wu *et al.*, 2005). In order to develop endometriosis lesions, ectopic endometrial cells must evade immune clearance in the peritoneal cavity. It is possible that de-regulation of immune cell function in the peritoneal cavity in endometriosis patients leads to an immunosuppressed environment that is permissive to the survival of refluxed endometrial tissue during retrograde menstruation. It is hypothesised that this can lead to the subsequent invasion of the peritoneal wall and formation of lesions. However, whether immune cell dysregulation is a pre-requisite for disease development or is a consequence of the presence of lesions in the peritoneal cavity is unknown (Izumi *et al.*, 2018). A correlation between endometriosis and autoimmune disorders such as inflammatory bowel disease and multiple sclerosis exists, suggesting that global immune cell changes in women with endometriosis could predispose to development of immune-mediated diseases (Jess *et al.*, 2012; Tiniakou, Costenbader and Kriegel, 2013).

1.1.2.6 Hormonal regulation

Endometriosis is known to be regulated by ovarian sex steroids. Early age at menarche is a risk factor for development of endometriosis, suggesting increased exposure to estrogen may incur increased risk of disease (Nnoaham *et al.*, 2012). Endometriosis lesions over-express aromatase, a steroidogenic enzyme required for estrogen synthesis (Zeitoun *et al.*, 1998). In a co-cultures system, estradiol increases the production of prostaglandin-E_2 in endometrial stromal cells. Prostaglandin-E_2 in turn increases aromatase activity through cAMP dependent activation of the aromatase promote II, further increasing estradiol levels (Noble *et al.*, 1997). This positive feedback loop creates an estrogen-rich local environment. In a baboon

model of endometriosis, aromatase inhibition attenuated peritoneal lesion growth, demonstrating the importance of local estrogen synthesis in peritoneal lesion development (Langoi *et al.*, 2013). Similarly, in a mouse model of surgically induced peritoneal endometriosis, estradiol treatment significantly increased lesion size (Zhao *et al.*, 2014). Treatment with an ERβ antagonist inhibited lesion growth in a mouse model of endometriosis, suggesting that the pro-proliferative effects of estradiol are at least in part mediated through ERβ (Han *et al.*, 2015). Interestingly, macrophages in endometriosis lesions in a pre-clinical mouse model predominantly expressed ERβ (Greaves *et al.*, 2015). Estrogen has been shown to have a pro-proliferative effect on immune cells such as macrophages, also modulating their phenotype and function (Pepe *et al.*, 2017), suggesting an interplay between estrogen and immune cell dysregulation may exist in endometriosis. This link has been demonstrated in inflammatory diseases such as asthma, where estrogen signalling has been shown to modulate allergic inflammation in the lung (Keselman and Heller, 2015; Keselman *et al.*, 2017).

Ectopic endometrial tissue also exhibits progesterone resistance (Attia *et al.*, 2000). Endometriotic lesions have reduced progesterone receptor expression when compared with eutopic endometrium, and have an absence of progesterone receptor-B (Attia *et al.*, 2000). In the eutopic endometrium, progesterone signalling normally stimulates epithelial cells to produce 17β-hydroxysteriod dehydrogenase (17β-HSD), which metabolises estradiol (He *et al.*, 2016). In the absence of progesterone receptors, 17β-HSD-2 is not synthesized and thus estradiol cannot be metabolised and levels remain high, in turn stimulating the growth of lesions (Noble *et al.*, 1997; Zeitoun *et al.*, 1998; Osiński *et al.*, 2018).

Taking this evidence together, it seems likely that endometriosis aetiology is a complex interaction between many different risk factors. Differential risk factors may be implicated in different forms of disease, however current understanding of these factors is poorly understood.

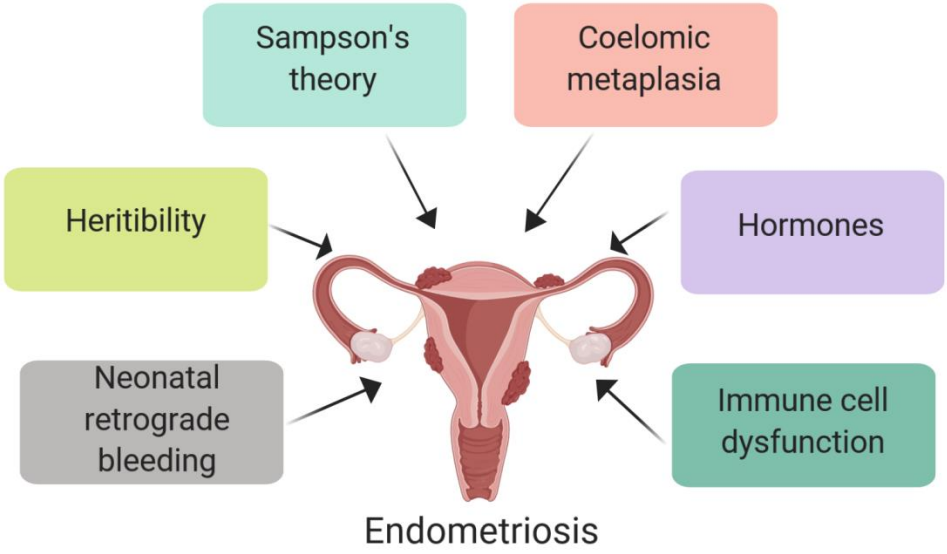


Figure 1.2. Theories for the aetiology of endometriosis

1.1.3 Pre-clinical mouse models for studying endometriosis

Pre-clinical models of endometriosis are an important platform required for studies into endometriosis pathophysiology. *In vivo* models are an attractive option for research aimed at understanding disease pathogenesis. They offer the ability to test therapeutic compounds, measure functional outcomes and have increased translational potential compared with an *in vitro* setting, dependent on the research question being investigated. Spontaneous endometriosis only occurs in humans and higher primates including baboons (Merrill, 1968) and rhesus monkeys (MacKenzie and Casey, 1975). Higher primate models of endometriosis are utilised, however due to cost and ethical concerns, mouse models are more frequently used. The high number of transgenic lines available in mice also makes them an ideal platform for mechanistic studies. However, mice do not menstruate or naturally develop endometriosis, thus endometriosis must be artificially induced. One exception which must be noted is the spiny mouse, which has been shown to menstruate and therefore offers a convenient non-primate model of menstruation, though research in this species is relatively new (Bellofiore *et al.*, 2017). Below is a summary of some of the more commonly utilised mouse models of endometriosis in common laboratory strains.

1.1.3.1 Heterologous mouse models

Heterologous mouse models are based on the transplantation of human tissue into nude mice with a mutation in forkhead box protein N1 (*FOXN1)* or severe combined immunodeficient mice which are homozygous for DNA-dependent protein kinase (*Prkdc)*. Transplantation of human tissue into these immunocompromised mice permits the survival and growth of human tissue without mounting a significant immune response. The ability to propagate patient samples allows functional studies to be performed in an *in vivo* setting but with human tissue, thus providing translational benefit over the use of mouse tissues. These models commonly involve transplanting human endometrial tissue either into the peritoneal cavity (Bruner *et al.*, 1997) or subcutaneously (Hull *et al.*, 2012). Immortalized human epithelial and stromal cell lines can also be utilised in a similar model system (Banu *et al.*, 2009). Whilst these mouse models are useful pre-clinical platforms, the requirement for immunodeficient mice makes these models problematic for studying the immune system and the absence of specific immune cells may affect disease pathophysiology.

1.1.3.2 Autologous mouse models

In the autologous mouse model of endometriosis, one horn of the uterus is ligated, removed and then cut into sections and sutured onto the peritoneal cavity or mesenteric cascade of the same mouse (Bilotas *et al.*, 2010). This process is beneficial as it negates a rejection response in the peritoneal cavity. Endometriosis-like lesions formed in this model are of a pre-defined size and location, making this model an ideal platform for testing therapeutic compounds or mechanistic studies which rely on lesion size as their primary outcome. This increases reproducibility across studies and more accurately determines the effect of a treatment on disease development. However, transplantation of full thickness uterus including myometrium is less reflective of endometriosis lesions seen in women, when we consider the retrograde menstruation theory where only the endometrial lining is refluxed into the peritoneal cavity (Sampson, 1927). Suturing of the uterus in the peritoneum also creates artificial inflammation, which may cause changes in immune cells and create artefacts which are in fact attributed to the suturing process and not the presence of disease.

1.1.3.3 Homologous mouse models

Homologous (syngenic) models of endometriosis involve isolation of the endometrial lining from the uterus and injection of endometrial tissue fragments into the peritoneal cavity of a recipient mouse (Capobianco *et al.*, 2011; Yuan *et al.*, 2017a). Mice are commonly supplemented with estrogen prior to collection of endometrial tissue (Yuan *et al.*, 2017a) or tissue is collected during estrus (Burns *et al.*, 2012), in the knowledge that estrogen is known to stimulate the growth of lesions (Zhao *et al.*, 2014). This represents a more physiologically relevant model as it mimics the dissemination of endometrial cells into the peritoneal cavity. The use of a donor and recipient mouse also allows mechanistic studies where either the donor or recipient may be genetically modified, also permitting lineage tracing studies. However, this model does not account for the fact that mouse endometrial tissue may not reflect the true cellular complement of refluxed menses endometrial tissue in women. Thus, the endometrial tissue used in this model may induces a differential immune response within the peritoneal cavity, a factor which may influence the development of endometriosis-like lesions in this model, forming lesions which may have a different cellular make-up to those observed in patients.

In order to produce a model that aims to recapitulate the process of retrograde menstruation, the Edinburgh team developed a unique mouse model of endometriosis where an artificial menstruation-like event is induced in donor mice. This generates 'menses-like' tissue which can then be collected and injected into the peritoneal cavity of recipient mice (Greaves *et al.*, 2014). Artificial menstruation in mice is achieved through the removal of the ovaries to deplete circulating hormone levels and subsequent exposure to a hormonal schedule which mimics a truncated human menstrual cycle (Cousins *et al.*, 2016). Mice first receive estradiol injections which mimic the estrogen dominated phase of the menstrual cycle. The mice then receive a progesterone implant which increases global progesterone levels, mimicking the progesterone dominated phase of the human menstrual cycle, as well as a decidualisation stimulus into the uterus, in the form of an intrauterine oil injection. Progesterone withdrawal is then stimulated by removal of the progesterone implant, followed by concomitant endometrial breakdown. The endometrial lining is then

scraped away from the myometrial wall and injected into the peritoneal cavity of a recipient mouse (Greaves *et al.*, 2014).

This model represents an ideal platform for studying endometriosis pathophysiology as it more closely mimics retrograde menstruation, the most widely accepted aetiology of endometriosis. It negates the need to suture endometrial fragments and bypassing any creation of artificial inflammation. The injection of tissue into the peritoneal cavity means lesions may form at a more 'natural' location rather than a pre-determined one (as with suturing) and the syngenic design also allows genetic manipulation of either the donor or recipient mouse for mechanistic and lineage tracing studies. Endometriosis lesions produced using this model are vascularised, innervated and infiltrated by macrophages, features of endometriosis lesions in women (Greaves *et al.*, 2014).

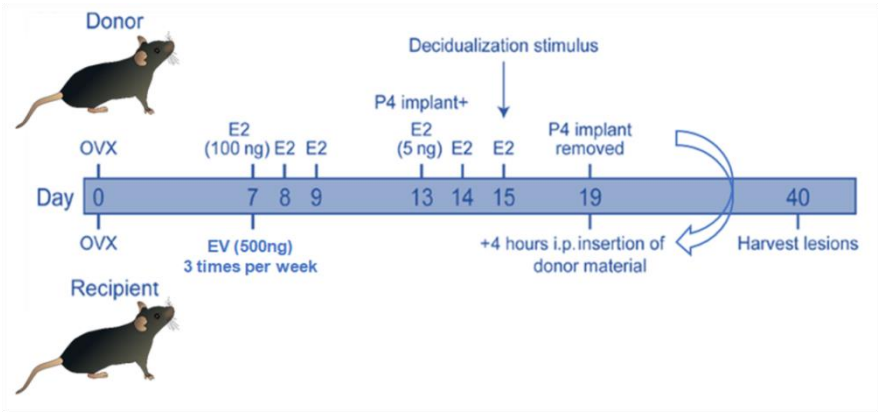


Figure 1.3. Schematic demonstrating timings and procedures of the Edinburgh mouse model of endometriosis*. Donor mice are ovariectomized and receive hormonal treatments which mimic the estrogen dominated (proliferative) and progesterone dominated (secretory) phases of the human menstrual cycle, producing mouse 'menses'-like endometrium for injection into the peritoneal cavity of recipient mice, which develop endometriosis lesions that phenocopy those seen in women* (Greaves *et al.*, 2014).

1.2 Macrophages have diverse phenotypes and functions

1.2.1 Macrophages are mononuclear phagocytes

Macrophages are immune cells which belong to the mononuclear phagocyte system, a family of professional phagocytes which includes dendritic cells and monocytes. Macrophages are able to respond to inflammatory events in the body and mount an

innate immune response, phagocytosing pathogens, apoptotic cells and debris. They are also important for antigen presentation and secretion of cytokines/chemokines to modulate the function of other immune cell subsets in the body, either in a pro or anti-inflammatory manner. Tissue macrophages colonise specific tissues in the body and are specially adapted to perform homeostatic functions within their tissue niche. For example, macrophages in the central nervous system (microglia) are crucial for myelination of neurons and shape neuronal development and function (Lloyd and Miron, 2016). Macrophages in the bone (osteoclasts) are specialised in bone resorption for bone remodelling (Bellido, 2014). Macrophages therefore play critical roles in maintaining tissue integrity and fighting infection in the body, and dysregulation of macrophage function has been linked to a number of autoimmune and chronic inflammatory diseases such as multiple sclerosis(Bogie, Stinissen and Hendriks, 2014), rheumatoid arthritis (Udalova, Mantovani and Feldmann, 2016), fibrosis (Wang *et al.*, 2017) and cancer (Noy and Pollard, 2014).

1.2.2 Macrophage ontogeny

Macrophages are derived from three key populations; macrophages originating from the yolk sack of the embryo, from the foetal liver and in adults, haematopoiesis in the bone marrow. The earliest macrophages arise from erythro-myeloid progenitors (EMPs) produced during primitive haematopoiesis in the extra-embryonic yolk sack at embryonic day (E)7.5 and 8.25. After blood circulation is established, EMP derived macrophages seed foetal tissue. Excluding microglia, these macrophages are partially or fully replaced by monocytes originating from the foetal liver, which differentiate into macrophages in tissues. Foetal liver monocytes are derived from either haematopoietic stem cells or EMPs generated in the yolk sack or the placenta or umbilical cord. Foetal liver monocyte-derived macrophages can persist into adulthood and form the tissue resident macrophage population, undergoing self-renewal, for example in the peritoneum, spleen, lung, skin and liver. In other organs, tissue macrophages derived from foetal liver monocytes are gradually replaced by recruited monocytes from the bone marrow. This process occurs in tissues such as the gut and dermis (Schulz *et al.*, 2012; Hashimoto *et al.*, 2013; Gomez Perdiguero *et al.*, 2015; Hoeffel *et al.*, 2015; Mass *et al.*, 2016).

Moreover, haematopoiesis in the bone marrow begins at around day E17.5. The bone marrow provides a niche for haematopoietic stem cells, where they undergo symmetrical division to autonomously self-renew or, in response to environmental cues, perform asymmetrical division producing lineage specific cells which include common myeloid progenitor cells (Knoblich, 2001; Yamashita *et al.*, 2010). These common myeloid progenitor cells differentiate into granulocyte-macrophage progenitor cells, eventually giving rise to monocytes which can then leave the bone marrow and enter the circulation.

In humans, peripheral blood monocytes form three populations based on differential expression of CD14 and CD16. Populations of $CD14^{hi}$ $CD16^{lo}$ and $CD14^{lo}$ $CD16^{hi}$ cells predominate with an intermediate population of $CD14^{hi}$ $CD16^{hi}$ cells also present. The $CD14^{hi}$ $CD16^{lo}$ subset are the most abundant in the blood and are known as 'classical' monocytes. Under inflammatory conditions, classical monocytes can extravasate into tissues where they differentiate into macrophages or dendritic cells (Ginhoux and Jung, 2014) and fulfil functions such as clearance of apoptotic bodies, stimulating angiogenesis and restoring integrity of tissues (Geissmann *et al.*, 2010).$CD14^{lo}$ $CD16^{hi}$ cells constitute the non-classical monocytes. Non-classical monocytes also exhibit extravasation into tissues during inflammation, however this is seen later on in the inflammatory process and these monocytes exhibit a bias towards differentiating into wound-healing like macrophages (Olingy *et al.*, 2017). A key role of the non-classical monocyte population is to patrol the blood vessels along the endothelial cell layer, providing immunosurveillance of vasculature and the surrounding tissues (Auffray *et al.*, 2007). Classical monocytes also patrol tissues and play a homeostatic role in steady state conditions, without differentiating into macrophages (Jakubzick *et al.*, 2013). Moreover, classical and non-classical monocytes in humans are widely accepted to be analogous to $Ly6C^{hi}$ classical and $Ly6C^{lo}$ non-classical monocytes in mice and exhibit significant homology at transcriptional analysis (Randolph *et al.*, 2009; Ingersoll *et al.*, 2010). Classical monocytes can be classified as $Ly6C^{hi}$ $CX3CR1^{lo}$ $CD43^{lo}CCR2^{hi}$, and non-classical monocytes as $Ly6C^{lo}$ $CX3CR1^{hi}$ $CD43^{hi}CCR2^{lo}$, with all monocyte populations being $CD11b^{hi}$ $F4/80^{int}$ (Ginhoux and Jung, 2014). As in humans, classical monocytes

extravasate into tissues under inflammatory conditions and non-classical monocytes patrol blood vessels performing immunosurveillance (Auffray *et al.*, 2007). Under steady state conditions, $Ly6C^{hi}$ monocytes can down-regulate Ly6C and adopt a $Ly6C^{lo}$non-classical phenotype (Yona *et al.*, 2013). Extravasation of $Ly6C^{hi}$ monocytes into tissues in response to inflammation is mediated by chemokine (C-C motif) ligand 2 (CCL2) binding to its receptor, C-C chemokine receptor type 2 (CCR2). CCR2 knockout mice show inhibited recruitment of classical monocytes to sites of inflammation (Lu *et al.*, 2002). As endometriosis is known to stimulate chronic inflammation in the peritoneal cavity, $Ly6C^{hi}$ classical monocytes could play a role in endometriosis pathophysiology ,though their role in disease is currently unknown.

1.2.3 Macrophage phenotype

Macrophages respond to their local microenvironment and change both their transcriptome and phenotype in response to local signals (Wynn, Chawla and Pollard, 2013). Historically, macrophages have been divided into either 'M1' classically or 'M2' alternatively activated cells. Classically activated macrophages are associated with inflammation, and express numerous pro-inflammatory markers. M2 macrophages are associated with homeostasis, wound healing and immunomodulation (Mantovani *et al.*, 2004). *In vitro* studies commonly use stimulation with granulocyte-macrophage colony-stimulating factor (GM-CSF) and the cytokine IFN-γ (Interferon γ) to generate classically associated macrophages, and stimulation with macrophage colony-stimulating factor (M-CSF) and the cytokines interleukin-4 (IL-4) and interleukin-10 (IL-10) to generate alternatively activated macrophages (Rey-Giraud, Hafner and Ries, 2012). Whilst this classification system is a useful tool for identifying macrophages at extremes of activation, it is now appreciated that macrophages exhibit a broad spectrum of phenotypes that are tissue and disease specific, and the 'M1' and 'M2' system cannot truly recapitulate the complex myriad of local growth factors and cytokines which modulate macrophage phenotype where M1/M2 markers often co-exist (Qian and Pollard, 2010; Martinez and Gordon, 2014). Transcriptional analysis of mouse tissue resident macrophage

populations demonstrates minimal overlap in mRNA expression, reflecting a divergence in gene expression patterns (Gautiar *et al.*, 2012). This heterogeneity reflects the ability of macrophages to modulate their gene expression in response to local tissue signals, becoming specialised to their tissue niche, be that in a healthy or diseased state. In disease, macrophages may modulate their phenotype dependent on disease stage or severity, and the mechanisms behind this are crucial for understanding their exact role in pathogenesis. A prominent research drive therefore has been to define macrophage phenotypes in disease states, with the potential of modulating macrophage phenotype or specifically targeting dysregulated macrophages for clinical benefit (Johnson and Newby, 2009; Wynn, Chawla and Pollard, 2013; Noy and Pollard, 2014; Udalova, Mantovani and Feldmann, 2016).

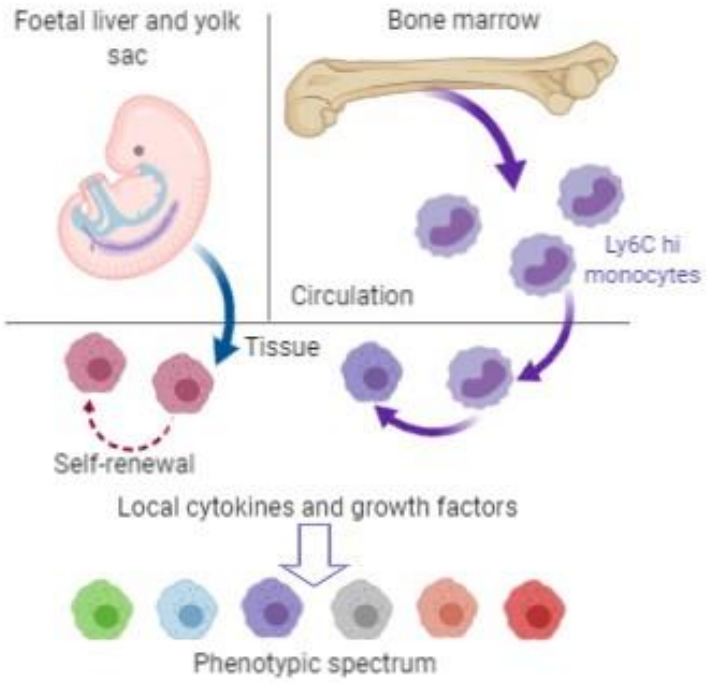


Figure 1.4. Macrophages are mononuclear phagocytes. *Tissue macrophages are seeded during foetal life from the foetal liver and yolk sack and undergo self-renewal. In adults, monocyte precursors extravasate from the bone marrow into the circulation, where they can then extravasate into tissues and differentiate into macrophages. In tissues, macrophages modulate their phenotype dependent on local cytokines and growth factors to specific tissue or disease associated phenotypes.*

1.2.4 Macrophages in the peritoneal cavity

1.2.4.1 Mouse

Peritoneal cavity macrophages are present in the peritoneal fluid which bathes peritoneal organs and are one of the most studied macrophage populations in mice, largely due to their ease of isolation. Two subsets of peritoneal macrophages are recognised in mice based on differential expression of F4/80 and MHC II. The tissue resident, so called 'large' (due to their larger size) peritoneal macrophages (LPM) are F4/80^{hi}, MHC II^{lo} and the monocyte-derived 'small' peritoneal macrophages (SPM) are F4/80^{lo} MHC II^{hi} (Ghosn *et al.*, 2010). F4/80^{hi}, MHC II^{lo} LPM are the most abundant macrophage population in the peritoneal cavity at steady state and form the tissue resident population. LPM are highly phagocytic and are important for immunosurveillance and homeostasis in the peritoneal cavity (Ghosn *et al.*, 2010) and for mediating recruitment and maintenance of B1 B cells, as well as being linked to regulation of intestinal immunity (Jackson-Jones and Bénézech, 2018). LPM undergo self-renewal in the peritoneal cavity. The proliferative capacity of LPM is regulated by GATA-binding factor 6 (Gata6), a transcription factor uniquely expressed by LPM in the peritoneal cavity, which also regulates macrophage phenotype (Rosas *et al.*, 2014). In mice, LPM consist of primarily embryonic-derived cells, however monocyte-derived macrophages replace embryonic-derived LPM over time, a process which is highly sex and age dependent, and slower in females. Ly6C^{hi} monocytes enter the peritoneal cavity in a CCR2-dependent manner and differentiate into a transient F4/80^{lo} MHC II^{hi} population (SPM), before differentiating into tissue resident F4/80^{hi} MHC II^{lo} cells (Bain *et al.*, 2016). Thus the LPM constitute both embryonic and monocyte-derived cells whereby these two populations have been shown to be transcriptionally distinct from each other (Bain *et al.*, 2016).

Whilst LPM in the peritoneal cavity are highly characterised, the heterogeneity and complement of the SPM population is less well defined. Commonly defined as F4/80^{lo} MHC II^{hi}, SPM can be sub-divided based on expression of CD11c and CSF1R into three populations, CD11c^{hi} CSF1R^{hi}, CD11c^{hi} CSF1R^{lo} and CD11c^{lo} CSF1R^{hi} (Bain *et al.*, 2016). Differentiation into SPM is regulated by the transcription factor interferon regulatory factor 4 (IRF4) (Kim *et al.*, 2016). Bain *et*

al demonstrated that production of the immuno-regulatory cytokine resistin-like molecule (RELM)α and expression of CD206 (mannose receptor) were markers of the SPM population in the peritoneal cavity (Bain *et al.*, 2016). SPM are implicated in the inflammatory response in the peritoneal cavity which is discussed in section 1.2.5 below, however their role in the steady state peritoneal cavity remains unclear (Cassado, D'Império Lima and Bortoluci, 2015).

1.2.4.2 Human

In humans, macrophages constitute 50% of peritoneal cavity leukocytes (Kubicka *et al.*, 1996). Tissue resident peritoneal macrophages have been defined by high expression of complement receptor of the immunoglobulin superfamily (CRIg) and low expression of CCR2. These cells are highly phagocytic and more numerous in steady state, also displaying similar transcriptional profiles to the mouse LPM population (Irvine *et al.*, 2016). Human monocyte-derived macrophages in the peritoneal cavity, anologous to F4/80^{lo} MHC II^{hi} SPM in the mouse, have been defined as CRIg^{lo}, CCR2^{hi}. This CRIg^{lo}, CCR2^{hi} population in humans has a reduced phagocytic capacity and is lower in number compared to CRIg^{hi} CCR2^{lo} tissue macrophages, consistent with characteristics of SPM in mice. It must be noted however that in humans, Gata6 was found to be more highly up-regulated in the pro-inflammatory CRIg^{lo} CCR2^{hi} population (Irvine *et al.*, 2016), highlighting that key differences between human and mouse peritoneal macrophages exist, and further research is critically required to clarify these differences.

1.2.5 Peritoneal cavity macrophages in inflammation

Under inflammatory conditions, LPM respond to inflammatory stimuli in a phenomenon known as the macrophage disappearance reaction (Barth *et al.*, 1995). In mice the LPM compartment undergoes a dramatic reduction in peritoneal cavity numbers largely by migration to the omentum, mediated by retinoic acid and Gata6 (Okabe and Medzhitov, 2014). Mechanistic studies in human are challenging and therefore knowledge of this physiological process in humans is minimal. Our knowledge of this process therefore stems largely from mouse models which will be the focus of this section. The degree of loss in the LPM population is highly

dependent on the dose of inflammatory stimuli and has been studied in a number of inflammatory models in mice, such as LPS, zymosan or thioglycollate induced peritonitis (Davies *et al.*, 2011; Gautier *et al.*, 2013; Liao *et al.*, 2016). LPM which persist during inflammation have been hypothesised to play a regulatory role in the peritoneal cavity by secretion of IL-10, an anti-inflammatory cytokine which has also been shown to regulate inflammatory SPM number (Liao *et al.*, 2016). LPM also play a key role in clearance of apoptotic cells during inflammation (Gautier *et al.*, 2013), demonstrating high expression of T-cell immunoglobulin and mucin domain containing 4 (Tim4) which recognises phosphatidyl-serine on apoptotic cell bodies (Wong *et al.*, 2010). Upon resolution of inflammation, the reduced LPM peritoneal population increases its proliferative capacity through a colony stimulating factor 1 receptor (CSF1R) mediated mechanism to restore LPM number (Davies *et al.*, 2011). Interestingly, LPM have been shown to infiltrate the liver by a non-vascular route in response to the damage-associated molecular pattern molecule (DAMP) ATP, where they play a key role in regeneration and tissue repair in the liver after sterile injury, modulating their phenotype in response to local tissue microenvironmental cues (Wang and Kubes, 2016). This migration implies that LPM have the ability to execute wound repair and tissue regeneration in visceral organs, however it is unknown whether LPM infiltrate or play a role in endometriosis lesions. The abundance and behaviour of LPM in women with endometriosis is unknown. However, as women with endometriosis experience inflammation in the peritoneal cavity and inefficient clearance of refluxed endometrial tissue, it is possible that perturbations of LPM exist that create a permissive environment for the formation of lesions. These mechanisms however have not been explored.

Furthermore, with a reduction of LPM numbers a concurrent increase in SPM and inflammatory Ly6C^{hi} monocytes is observed in a number of mouse models of peritoneal inflammation (Barth *et al.*, 1995). SPM exhibit a pro-inflammatory response when challenged with LPS *in vitro*, producing high levels of chemokine (C-C motif) ligand 5 (CCL5), chemokine (C-C motif) ligand 3 (CCL3) and tumor necrosis factor-α (TNF-α), as opposed to LPM which produce G-CSF and GM-CSF under LPS stimuli (Cassado, D'Império Lima and Bortoluci, 2015). In an *in vivo*

model of peritonitis, SPM also produce high amounts of pro-inflammatory cytokines including TNF-α, interleukin-1β (IL-1β) and IFN-γ (Rückerl*et al.*, 2017), and are critical for clearance of bacterial infection in the peritoneal cavity after bacterial challenge in the mouse (Cassado *et al.*, 2011). The ability of SPM to respond to inflammatory stimuli by producing pro-inflammatory cytokines enables rapid response to immunological challenge in the peritoneal cavity. At resolution of inflammation, SPM have been shown to undergo apoptosis (Gautier *et al.*, 2013) but can also migrate to local draining lymph nodes (Bellingan *et al.*, 1996). SPM however have also been shown to persist in the cavity and can eventually differentiate into F4/80^{hi} MHC II^{lo} cells (Yona *et al.*, 2013), suggesting that inflammation has the potential to alter the complement of peritoneal cavity macrophage populations, even after homeostasis has been restored. The multiple fates of SPM reflect the heterogeneity in this cell compartment, and the roles of SPM sub-populations in inflammation are still largely undefined. The SPM composition in women with endometriosis has not been investigated, and although peritoneal macrophage depletion leads to decreased lesion formation in mice (Bacci *et al.*, 2009), the constitution and roles of peritoneal macrophage sub-populations in endometriosis has not been assessed.

1.5 Macrophages play diverse roles in health and disease

Macrophages have been shown to play instrumental roles in development, maintenance and progression of a number of diseases (Wynn, Chawla and Pollard, 2013). Peritoneal cavity macrophage perturbations and dysregulation are linked to a number of adverse clinical outcomes. An increase in peritoneal macrophages was associated with negative outcomes in patients with peritonitis (Liao *et al.*, 2017), and dysregulation of peritoneal macrophages has been linked to acute pancreatitis, where peritoneal macrophages produce increased levels of pro-inflammatory cytokines to exacerbate disease (Shrivastava and Bhatia, 2010). Macrophages have also been implicated in the formation of adhesions, a common complication after abdominal surgery (Burnett *et al.*, 2006). Peritoneal cavity macrophages are therefore intricately linked to disease in the peritoneal cavity in humans, including in endometriosis patients (Haney, Muscato and Weinberg, 1981).

Although endometriosis is a benign disease a number of parallels have been drawn between the condition and cancer. Macrophages are unambiguously at the centre of the pathophysiology of both diseases. The significant progress in cancer-associated macrophage research therefore serves as an ideal platform to inform studies within the endometriosis field.

Macrophage infiltration in tumours has been shown to be a predictor of poor clinical outcomes in malignancy (Zhang *et al.*, 2012; Candido and Hagemann, 2013), attributed to the fact that macrophages promote initiation, progression and metastasis in most cancers (Qian and Pollard, 2010). In the last decade a major focus has been to define macrophage populations which constitute the tumour resident population. In a mouse model of breast cancer, $Ly6C^{hi}$ inflammatory monocytes are recruited to metastatic sites in a CCR2/CCL2 mediated mechanism to form tumour associated macrophages (TAMs). Inhibition of CCL2/CCR2 signalling with an anti-CCL2 antibody inhibited monocyte recruitment thereby inhibiting metastasis and prolonging survival of the mice (Qian *et al.*, 2011). Similarly, mouse models of Lewis lung carcinoma demonstrated that TAMs were derived from CCR2 driven recruitment of $Ly6C^{hi}$ monocytes and blockage of CCL2 decreased tumour growth (Sawanobori *et al.*, 2008; Cortez-Retamozo *et al.*, 2012). Depletion of Tie2+ monocytes/macrophages in nude mice harbouring human glioma tumours was demonstrated to cause tumour regression (De Palma *et al.*, 2005). The role of $Ly6C^{hi}$ monocytes is therefore instrumental in a number of cancers and the CCR2/CCL2 axis has been implicated in many instances, particularly in metastatic disease (Lim *et al.*, 2016). Notably, women with endometriosis show an up-regulation of CCL2 in peritoneal fluid and ectopic endometrial cells isolated from lesions over-produce the CCL2 ligand (Pizzo *et al.*, 2002; Li *et al.*, 2012). Whether or not this recruitment pathway is implicated in endometriosis is currently unknown.

Furthermore, tissue resident macrophages have also been implicated in cancer pathophysiology and can contribute to the TAM population. For example, in a mouse model of pancreatic ductal adenocarcinoma, Zhu *et al* demonstrated using a parabiosis model that TAMs were derived from both embryonically derived tissue resident macrophages as well as from circulating $Ly6C^{hi}$ monocytes. During tumour

development embryonically derived macrophages expanded via *in situ* proliferation and had a pro-fibrotic role in tumours. Use of a CSF1R neutralizing antibody alongside clodronate liposome treatment to deplete tissue resident macrophages lead to a reduction in tumour size and increased the survival of the mice. Monocyte-derived macrophages however played a key role in antigen presentation. Use of CCR2 knockout mice or a CCR2 inhibitor to inhibit recruitment of $Ly6C^{hi}$ monocytes did not affect tumour growth (Zhu *et al.*, 2017). This study highlights the importance of defining the ontogeny of TAMs in order to decipher which populations are fundamentally required for tumour growth, with the aim of improving clinical outcomes.

Whilst TAMs may have multiple origins, it has been demonstrated that the tumour microenvironment can modulate macrophage phenotype to promote malignancy, suggesting that origin may not wholly define phenotype when macrophages are exposed to cytokines and growth factors locally in the tumour. A number of different macrophage populations within tumours have been described which play differential roles and have different phenotypes. For example, populations of invasive macrophages, perivascular macrophages, metastasis associated macrophages, angiogenic macrophages (which notably are $Tie2^{+}$) and immunosuppressive macrophages which secrete high levels of IL-10 have been described (Qian and Pollard, 2010). Detailed profiling of hepatocellular carcinoma biopsies demonstrated the presence of various macrophage sub-types in tumours which had both pro and anti-tumoral properties (Wu and Zheng, 2012). It is unknown whether lesion resident macrophages in women with endometriosis exhibit phenotypic heterogeneity, as current studies have largely evaluated single activation markers in an immunohistochemical approach (Bacci *et al.*, 2009).

1.3 Macrophages in the endometrium

The endometrium undergoes cyclic proliferation, differentiation, shedding (menstruation) and repair in response to ovarian-derived estrogen and progesterone during the menstrual cycle. In the normal cycling endometrium, an influx of macrophages occurs during the secretory and menstrual phases, along with a concomitant increase in macrophage-derived cytokines and proteases (Critchley *et*

al., 2001). Evidence from a mouse model of menstruation identified an influx of classical monocytes which differentiated into macrophages in the endometrium during the repair phase of the menstrual cycle (Cousins *et al.*, 2016). Monocyte extravasation from blood vessels into the endometrium has been shown to be regulated by CCL2 (Arici, MacDonald and Casey, 1995; Jones, Kelly and Critchley, 1997) and CX3C chemokine receptor 1 (CX3CR1) (Kitaya*et al.*, 2004). The influx of macrophages into the endometrium is syngeneic to the plethora of key roles they are thought to play in modulating endometrial differentiation, breakdown and repair. During the proliferative phase macrophages have been postulated to play a role in regeneration and proliferation of the functional layer of the endometrium and express activation and adhesion markers CD54, CD69 and CD71 (Eidukaite and Tamosiunas, 2004). Macrophages play a role in initiating menstruation by secreting matrix metallopeptidases (MMPs) (Zhang *et al.*, 2005). Specifically, secretion of MMP-12, MMP-9 and MMP-14 are required for the breakdown of the functional layer of the endometrium during menstruation (Salamonsen, Zhang and Brasted, 2002; Curry and Osteen, 2003; Jeziorska *et al.*, 2004). Macrophages are also implicated in regulating gland remodelling and angiogenesis by production of vascular endothelial growth factor (VEGF) (Sharkey *et al.*, 2000; Garry *et al.*, 2010). Macrophages are therefore key players in augmenting dynamic remodelling and repair in the endometrium.

In addition, regulation of endometrial macrophage function is also in part achieved by hormone signalling. Endometrial macrophages express estrogen receptor-β (ER-β) (Bombail *et al.*, 2008). In response to estrogen, macrophages increase their proliferative capacity and undergo phenotypic changes to adopt a phenotype which represents a more 'wound healing-like' population (Pepe *et al.*, 2017). Estrogen signalling can therefore accelerate the wound healing process and this is in part instrumented by increasing the production of macrophage derived proteases, MMPs, fibroblast growth factor, VEGF and cytokines such as resistin like alpha (RELMα) (McLaren *et al.*, 1996; Rochefort *et al.*, 2001; Campbell *et al.*, 2014; Pepe *et al.*, 2017). Interestingly, growth of endometriosis lesions is stimulated in mice by treatment with estrogen (Zhao *et al.*, 2014), and growth is also inhibited by treatment with an ERβ antagonist (Han *et al.*, 2015), suggesting that mechanisms active within the endometrium which stimulate tissue repair and remodelling may also play key

roles in modulating the growth and development of endometriosis lesions. Endometrial macrophages do not express the progesterone receptor (Stewart, Bulmer and Murdoch, 1998), however macrophage gene expression is significantly altered in response to progesterone (Cheng *et al.*, 2007), suggesting an indirect method of regulation. Interestingly, exposure to cortisol was demonstrated to increase expression of angiogenic genes such as CXCL2, CXCL8, and VEGFC in macrophages *in vitro*, suggesting that local cortisol levels could be important for regulating angiogenesis within the remodelling endometrium (Thiruchelvam *et al.*, 2016). Macrophages are therefore intricately involved in physiological changes occurring in the endometrium and this is regulated by exposure to local cytokines, growth factors and hormones which modulate their phenotype, function and recruitment throughout the menstrual cycle.

1.4 The role of macrophages in endometriosis

1.4.1 Macrophages are involved in the growth, vascularisation and innervation of lesions

Endometriosis lesions from women are highly infiltrated by CD68+ macrophages which are present within the stroma of the tissue and can also be found in close proximity to glands (Bacci *et al.*, 2009; Greaves *et al.*, 2014). Patients have an increased number of peritoneal macrophages (Haney, Muscato and Weinberg, 1981) which exhibit a dysregulated phenotype. In women with endometriosis, peritoneal macrophages have reduced phagocytic capacity due to low levels and activity of matrix metalloproteinase 9, which is required for extracellular matrix degradation and is regulated by prostaglandin E2 (PGE2) (Wu *et al.*, 2005). In a co-culture system, in the presence of endometrial stromal cells isolated from ectopic endometrial tissues, monocyte-derived macrophages secreted IL-10 and TGF-β, which in turn suppressed cytotoxicity and viability of NK cells (Yang *et al.*, 2017), suggesting that macrophages are immunosuppressive in the presence of ectopic endometrial stromal cells and can act to suppress NK cells in the peritoneal cavity. Whilst peritoneal macrophages are dysregulated in women with endometriosis, endometrial macrophages have also been shown to exhibit differential properties. Reflecting on the theory of retrograde menstruation, where endometrial tissue is

refluxed into the peritoneal cavity to form lesions in some women (Sampson, 1927) the presence of macrophages in refluxed endometrial tissue in women has the potential to augment disease development in the peritoneal cavity. A number of studies have demonstrated perturbations in macrophage populations in the eutopic endometrium of endometriosis patients. Women with endometriosis have more endometrial macrophages which express lower levels of the 'wound-healing' marker CD163 compared to women without disease, however the exact mechanisms behind these alterations are unknown (Berbic *et al.*, 2009; Takebayashi *et al.*, 2015). Analogous to this, increased levels of CCL2 can be observed in the endometrium of women with disease which corresponds to disease severity, suggesting increased influx of monocytes in disease (Jolicoeur *et al.*, 1998). Increased matrix metalloproteinase-9 (MMP-9) co-localised with $CD68^{+}$ macrophages in the endometrium of women with endometriosis is indicative of an increase in the number of macrophages implicated in tissue remodelling. This was suggested to possibly enhance the ability of ectopic endometrial tissue deposits to implant in the peritoneal cavity (Collette *et al.*, 2006). Whilst evidence of macrophage dysregulation in the eutopic endometrium of women with endometriosis exists, the role of endometrial macrophages in endometriosis has not been defined as functional studies in this area are lacking.

Studies in women have strongly implied a role for macrophages in endometriosis, however mechanistic studies, mostly in mouse models, have significantly improved our understanding of macrophage biology in disease. Studies to date have largely focussed on defining the role of macrophages in syngenic mouse models using various cell depletion approaches. A commonly utilised depletion method uses liposomes encapsulating bisphonates. These liposomes are taken up by phagocytic cells which are then degraded within the cell, releasing bisphonate and causing subsequent cell death. This method therefore selectively depletes phagocytic cells and is non-toxic to non-phagocytic cells, and has been commonly used to deplete phagocytic macrophage populations (Rooijen and Sanders, 1994). In a syngenic mouse model of disease, Bacci *et al* used clodronate liposomes and a monoclonal anti-F4/80 antibody to deplete/inhibit peritoneal macrophage function in mice with

induced endometriosis and demonstrated that both treatments caused a reduction in growth and blood vessel formation in lesions (Bacci *et al.*, 2009). Adoptive transfer of *in vitro* generated 'pro-inflammatory' (stimulated with IFN-γ), 'anti-inflammatory' (stimulated with macrophage-colony-stimulating factor and IL-10) or 'non-polarized' (stimulated with macrophage-colony-stimulating factor) macrophages lead to differential effects on lesion development. 'Non-polarized' macrophages had no effect on lesion number or weight, however adoptive transfer of pro-inflammatory macrophages reduced lesion weight. Conversely, adoptive transfer of anti-inflammatory macrophages caused an increase in lesion weight. The authors noted that lesion architecture was also disrupted in mice which had received adoptive transfer of pro-inflammatory macrophages (Bacci *et al.*, 2009). Together, this data suggests that anti-inflammatory macrophages may be important for the growth and development of lesions and pro-inflammatory macrophages have an antagonistic effect, clearing ectopic endometrial tissue and disrupting lesion architecture. Whilst this data provides an important insight into the roles of macrophage phenotypes in endometriosis, the use of the M1/M2 paradigm is perhaps a slightly simplistic approach and the exact phenotype of macrophages in endometriosis and their role in disease is currently unknown. Capobianco *et al* identified Tie-2 expressing macrophages that infiltrated mouse and human lesions. Depletion of Tie-2^+ macrophages was achieved using a bone marrow chimera from mice expressing a suicide gene (herpes simplex virus type 1 thymidine kinase) expressed under control of the Tie2 promoter into wild-type mice. After treatment with ganciclovir (an anti-viral drug), bone-marrow derived $Tie2^+$ cells were selectively depleted and growth of endometriosis lesions was inhibited, with loss of neovascularisation and glandular organisation in the resultant lesions (Capobianco *et al.*, 2011). Sekiguchi *et al* demonstrated that VEGFR1 knockout mice had smaller and less vascularised lesions than WT in a mouse model where sections of uterus were sutured onto the peritoneal wall. Using bone marrow chimeras they demonstrated that $VEGFR1^+$ cells in lesions were bone marrow derived $CD11b^+$ macrophages, although the authors did not account for the fact that CD11b can also be expressed on monocytes, neutrophils, eosinophils and some subsets of dendritic cells (Hey, Tan and O'Neill, 2016). WT endometriosis mice were also treated with clophosome N which depleted phagocytic

cells in the peritoneal cavity at the time of endometriosis induction, and demonstrated that growth and angiogenesis in lesions was reduced (Sekiguchi *et al.*, 2019a). A similar study using liposomal bisphosphonate to deplete phagocytic peritoneal populations also demonstrated reduced growth of endometriosis lesions in a rat model (Haber *et al.*, 2009). It seems clear therefore that in experimental models of endometriosis, depletion of peritoneal phagocytic macrophage populations inhibits growth and angiogenesis of induced lesions.

In vitro studies have aimed at assessing the interaction between endometrial stromal cells and macrophages in disease. In a co-culture system, culture of endometrial stromal cells with autologous macrophages isolated from women with endometriosis increased the invasive and clonogenic ability of stromal cells (Chan *et al.*, 2017). Co-culture with ectopic endometrial stromal cells was also shown to decrease the phagocytic capacity of macrophages and increased the survival and proliferation of stromal cells compared to eutopic endometrial stromal cells in a study by Mei *et al*(Mei, Chang and Sun, 2017). A similar effect was also reported by Shao *et al* (Shao *et al.*, 2016). Reciprocal signalling therefore appears to be occurring between ectopic endometrial stromal cells and macrophages, which could contribute to their survival and the formation of endometriosis lesions in the peritoneal cavity, however the precise mechanisms are yet to be elucidated and the specific macrophage populations involved are unknown.

Furthermore, a role for macrophages in neurogenesis in endometriosis lesions has been established in the literature, suggesting a role for macrophages in the generation of endometriosis-associated pain. Indeed, nerve infiltration in lesions is positively correlated with higher reported pain scores in women (McKinnon *et al.*, 2012). Cholinergic, adrenergic, sensory Aδ and C nerve fibres have been identified in lesions (Tokushige *et al.*, 2006; Arnold *et al.*, 2012), and macrophages are densely populated in areas of high nerve density (Tran *et al.*, 2009; Greaves *et al.*, 2015). Greaves *et al* reported that in response to estradiol, nerve fibres secreted CCL2 and CSF-1 which attracted macrophages, which in turn secreted neurotrophin 3 and brain-derived neurotrophin factor, stimulating neurogenesis (Greaves *et al.*, 2015).

We have also recently published that macrophage-derived insulin-like growth factor-1 (IGF-1) is a key signal for nerve outgrowth and sensitization in endometriosis. Depletion of peritoneal macrophages by clodronate liposomes reversed abnormal pain behaviour in mice with induced endometriosis and notably reduced the number of lesions in the peritoneal cavity, providing a direct link between macrophages and endometriosis-associated pain/lesion development. Mechanistically we demonstrated that *in vitro*, macrophages treated with peritoneal fluid from women with endometriosis had an up regulation of IGF-1 at the mRNA level. Macrophage-derived IGF-1 increased the growth of embryonic rat dorsal root ganglion explants and this was reversed by an IGF-1 inhibitor. Similarly, we demonstrated that IGF-1 inhibition by the small molecule inhibitor linsitinib in our mouse model could reverse abnormal pain behaviours (Forster *et al*., 2019). Taken together, we demonstrated that macrophages are implicated in neurogenesis and the generation endometriosis-associated pain symptoms, and this is at least in part mediated by IGF-1.The reciprocal signalling that occurs between macrophages and nerve fibres therefore appears critical in regulating neurogenesis in lesions and neuroinflammation is a key driver of endometriosis pathophysiology.

1.4.2 Macrophage ontogeny and phenotype in endometriosis

Whilst a role for macrophages in endometriosis pathophysiology is established, the ontogeny and phenotype of endometriosis-associated macrophages is still poorly understood. Greaves *et al* demonstrated in a mouse model of endometriosis that lesion resident macrophages are derived from both the endometrium and infiltrating macrophage populations (Greaves *et al.*, 2014). These infiltrating macrophage populations are likely to constitute peritoneal or monocyte-derived macrophages infiltrating from the peritoneal cavity, however the exact origins of these populations is currently unknown. Using bone marrow chimeras Sekiguchi *et al* demonstrated that $CD11b^+$ cells from the bone marrow infiltrate and accumulate in endometriosis lesions in a mouse model (Sekiguchi *et al.*, 2019b). These cells could represent a monocyte/ macrophage population, however as mentioned previously, the possibility that $CD11b^+$ cells could be neutrophils, eosinophils and or certain subsets of dendritic cells was not taken into account (Hey, Tan and O'Neill, 2016). Capobianco

et al demonstrated that bone marrow derived $Tie2^+$ cells infiltrated endometriosis lesions in a mouse model, again demonstrating that bone marrow derived cells could infiltrate from blood vessels, however the possibility that other immune cells could express Tie2 was not taken into account (Capobianco *et al.*, 2011). It appears therefore that bone marrow derived monocytes/macrophages infiltrate endometriosis lesions, however current studies fail to specifically identify the infiltrating populations. Whether peritoneal macrophages (LPM and SPM) infiltrate lesions is currently unknown.

Furthermore, the phenotype of macrophages in endometriosis lesions has long been described as being wound healing and 'M2-like', however few studies have taken into consideration the complexities of macrophage phenotype, where pro-inflammatory and wound-healing like markers often co-exist in response to complex signals from the local tissue microenvironment (Martinez and Gordon, 2014). In humans, lesion resident macrophages express the scavenger receptors CD163 and CD206, associated with haemoglobin scavenging and silent clearance of debris (Bacci *et al.*, 2009). Cominelli *et al* also identified $CD163^+$ $CD206^+$ macrophages in superficial lesions from women, which also expressed high levels of matrix metalloproteinase-27, associated with tissue remodelling (Cominelli *et al*, 2014). Duan *et al* characterised nitric oxide synthase ($iNOS^+$) pro-inflammatory and $CD163^+$ wound healing-like macrophages in mouse endometriosis lesions (Duan *et al.*, 2018). In a rhesus macaque model of endometriosis, lesions were highly infiltrated by $CD163^+$ macrophages (Smith *et al.*, 2012). Johan *et al* examined infiltrating macrophage phenotype over time in endometriosis lesions in a heterologous mouse model and found that macrophage phenotype was progressively altered over time. Macrophages initially expressed pro-inflammatory markers iNOS and major histocompatibility complex II (MHC II), however at 7 and 14 days post lesion induction a higher proportion of macrophages expressed arginase 1 and CD204 (scavenger receptor A), which are more associated with a tissue remodelling phenotype (Johan *et al.*, 2019a). This study therefore demonstrates that macrophage phenotype in endometriosis lesions is dynamic and progressively changes as lesions develop in the peritoneal cavity. However, as with previous studies, the small number of markers assessed

makes it difficult to truly re-capitulate the complex phenotype of macrophages in the tissue. Whilst macrophages in endometriosis lesions possessing a 'wound healing' like phenotype is synergistic with their role in growth and angiogenesis in lesions, a more comprehensive analysis of macrophage phenotype in endometriosis lesions is required. It is also unknown whether different phenotypes exist within endometriosis lesions which could play differential roles in pathology, and identifying these populations is key for understanding which macrophage populations are driving pathology.

1.4.3 Cytokines and chemokines implicated in macrophage dysregulation in endometriosis

Perturbations in cytokine levels in peritoneal fluid, serum and ectopic endometrial tissue of women with endometriosis have been reported, possibly reflective of increases in peritoneal immune cell populations and dysregulation of their functions (May *et al.*, 2010; Borrelli, Abrão and Mechsner, 2014). A number of factors relate to macrophage function and some of the roles these cytokines/chemokines are currently known to play in inflammatory diseases and the proven or potential role they may play in endometriosis are summarized in *Table 1.1*. Whilst we know that women with endometriosis have differential cytokine profiles to women without disease, the roles these cytokines play in endometriosis is largely unknown. Cytokines which have been involved in functional studies include CCL2, IL-6 and IL-8. Ming-Qing *et al* demonstrated that ectopic endometrial stromal cells (lesions) isolated from women secreted more CCL2 compared to respective eutopic endometrial stromal cells, and that CCL2 was able to encourage survival and invasiveness of endometrial stromal cells *in vitro*, suggesting that CCL2 may mediate macrophage-stromal cell signalling to stimulate disease (Li *et al.*, 2012). In a similar manner, macrophages cultured in conditioned medium from cultured ectopic endometrial cells isolated from women secreted high levels of IL-6 which increased the migration of endometrial stromal cells, suggesting that IL-6 could also be playing a role in macrophage-stromal cell reciprocal signalling (Woo *et al.*, 2017). IL-8 is a chemokine secreted by macrophages which is chemotactic for neutrophils and was

shown to play a role in angiogenesis in a mouse model of endometriosis, whereby IL-8 signalling was mediated by hypoxia (Hsiao *et al.*, 2014).

Cytokines up-regulated in the peritoneal fluid of women with endometriosis are implicated in macrophage recruitment and survival. For example, CSF-1 signalling plays an instrumental role in eliciting and regulating tumour associated macrophages in a number of cancers (Hume and MacDonald, 2012), but there have been no studies on CSF-1 in endometriosis to date. CCL2 and CCL5 are also implicated in the recruitment of macrophages to breast cancer tumours (Svensson *et al.*, 2015), however we don't currently understand how macrophages are recruited to endometriosis lesions.

Cytokine	Dysregulated in endometriosis (human)	Mechanistic evidence from other inflammatory disorders (mouse models)	Proven/possible role in endometriosis
Chemokine (C-C motif) ligand 2	↑ Peritoneal fluid ↑ Ectopic endometrial stromal cells	•Recruitment of TAMs in breast cancer (Svensson *et al*, 2015)	•Promoting survival and invasiveness of ectopic endometrial stromal cells (Li *et al*, 2012) •Macrophage recruitment
Chemokine (C-C motif) ligand 5	↑ Peritoneal fluid	•Recruitment of TAMs in breast cancer (Svensson *et al*, 2015)	•Macrophage recruitment
Interleukin-1	↑ Peritoneal fluid	• Macrophage-derived IL-1β increases breast cancer cell migration and adhesion (Holen *et al*, 2016)	• Increasing migration and adhesion of endometrial stromal cells
Interleukin-6	↑ Peritoneal fluid	•Mediates recruitment of TAMs in non-small cell lung cancer by up-regulating CCL2/CCL5 (Wang *et al*, 2017) •Modulates macrophage phenotype in gastric cancer (Fu *et al*, 2017)	•Stimulating migration of endometrial stromal cells (Woo *et al*, 2017) •Macrophage recruitment
Interleukin-8	↑ Peritoneal fluid	• Macrophage secreted IL-8 promotes migration and invasion of breast cancer cells (Cao *et al*, 2017)	• Promotes angiogenesis in lesions (Hsiao *et al*, 2014) • Promoting endometrial stromal cell survival and invasion
Interleukin-16	↑ Peritoneal fluid	• Mediates macrophage polarisation to a 'wound healing-like' phenotype (Huang *et al*, 2019)	• Modulating macrophage phenotype to promote disease
Tumour necrosis factor-α	↑ Peritoneal fluid	• Secreted by pro-inflammatory macrophages, modulates inflammatory responses (Valilou *et al*, 2018)	• Modulating response to inflammation in the peritoneal cavity
Colony-stimulating factor-1	↑Ectopic endometrial tissue	•Supporting proliferation of TAMs and increases tumour growth (Hume and MacDonald, 2012) •Modulates TAM phenotype in hepatocellular carcinoma (Ao *et al*, 2017)	•Supporting the survival and proliferation of macrophages
Interleukin-13	↓ Peritoneal fluid ↓ Serum	• Mediates macrophage phenotype and macrophage-mediated tissue repair (Bosurgi *et al*, 2017)	• Modulation of macrophage phenotype

Table 1.1. Cytokine and chemokine levels in endometriosis patients and their possible role in disease

Similarly, perturbations in cytokines and chemokines have also been reported in animal models of endometriosis. Of relevance to this thesis, Greaves *et al* reported that induced endometriosis lesions in the Edinburgh mouse model of endometriosis

show an up-regulation of the macrophage chemotactic factors CCL5 and CCL2 compared to adjacent healthy peritoneum (Greaves *et al.*, 2014). Notably, CCL2 is up-regulated both in women with disease and in the Edinburgh mouse model. In the future it will be important to understand the mechanisms which mediate macrophage recruitment and phenotypic modulation in disease as interference of these pathways may inhibit pro-disease macrophage populations and provide clinical benefit for women.

1.6 Macrophage targeted therapies

Macrophages offer an attractive therapeutic target due to their instrumental role in a number of pathologies (Noy and Pollard, 2014). Inhibition of macrophage signalling or recruitment, as well as re-education of disease-associated macrophages to a 'healthy' phenotype could be of clinical benefit to patients where macrophages are implicated in disease pathophysiology. Identification of pathological macrophage populations and a detailed understanding of their regulation, recruitment and phenotype is therefore a fundamental step before the development of therapeutics which specifically target disease-associated macrophages is possible.

Due to the intricate involvement of macrophages in many cancers, macrophage-targeted therapies have received much attention in the literature and a number of *in vivo* studies and clinical trials have demonstrated efficacy in using macrophage-mediated treatments to improve clinical outcomes (Noy and Pollard, 2014). A subset of studies have targeted proliferation of tumour associated macrophages in an effort to alleviate tumour burden and improve clinical outcomes. Strachen *et al* demonstrated that targeting the CSF1-receptor with a small molecule inhibitor attenuated the turnover rate of tumour-associated macrophages (TAMs) and decreased tumour growth in mouse models of breast and cervical cancer(Strachan *et al.*, 2013). A phase I trial demonstrated a significant reduction in macrophage number in solid tumours after anti-CSF1R treatment (Gomez-Roca *et al.*, 2019), and CSF1R inhibition showed an improvement in clinical outcomes including improvement of symptoms in patients with diffuse-type giant cell tumours (Ries *et al.*, 2014). Inhibiting macrophage proliferation therefore appears to be of clinical benefit in cancer models and subsets of cancer patients. Future treatments may

specifically target disease-associated macrophage populations, as CSF1 is a key regulator of macrophage proliferation and survival in most tissues (Stanley and Chitu, 2014). The proliferative capacity of endometriosis lesion-resident macrophages is currently unknown, thus further research is required to determine whether this treatment strategy would be of benefit to women with endometriosis.

Another potential mechanism of therapeutic intervention could involve blocking recruitment of pathogenic macrophage populations. TAMs in pancreatic ductal adenocarcinoma were shown to be recruited via a CCL2/CCR2 mechanism from the bone marrow (Mitchem *et al.*, 2013). A phase 1b trial in pancreatic cancer patients utilised a CCR2 inhibitor alongside chemotherapy and demonstrated a reduction in macrophage infiltration into tumours compared to chemotherapy alone (Wang-Gilliam*et al.*, 2015). Although this was only a small trial of 47 patients, it provides proof of concept that TAM infiltration can be inhibited in patients when the recruitment mechanisms of disease-associated macrophages are defined. A similar approach may therefore be useful in blocking infiltration of macrophages into endometriosis lesions, however the mechanisms which regulate recruitment into lesions are currently poorly understood.

Whilst progression in macrophage targeted therapies is promising, current therapies do not specifically target dysregulated macrophages but have the potential to affect macrophage populations throughout the whole body. For example, use of a CCR2 inhibitor in pancreatic cancer patients significantly reduced the number of circulating $CCR2^{+}$ inflammatory monocytes (Wang-Gilliam *et al.*, 2015). These immune modulating treatments have the potential to produce toxicity due to the global effects on the immune system. As our understanding of macrophages in disease progresses, it is increasingly recognised that macrophage origins and heterogeneity in disease are crucial areas of research before specific, targeted treatments can be designed (Wynn, Chawla and Pollard, 2013). A further body of research which more critically describes macrophage sub-populations, active recruitment mechanisms and phenotype in an endometriosis setting is therefore critically required before macrophage-targeted treatments may be a possibility for women with endometriosis.

1.7 Summary

Macrophages are abundant in endometriosis lesions and have been shown to be critical for the growth, development, vascularisation and innervation of lesions and generation of pain symptoms *in vivo* (Bacci *et al.*, 2009; Capobianco *et al.*, 2011; Greaves *et al.*, 2015; Forster *et al*., 2019). In the Edinburgh mouse model of endometriosis, endometrial macrophages persist in decidualised endometrial tissue and contribute to the lesion resident population, however the role of this population in lesion development is unknown. The majority of lesion macrophages are derived from infiltrating populations but the complement of these infiltrating populations remains un-defined (Greaves *et al.*, 2014). In cancer studies, differential targeting of recruited monocyte-derived or tissue resident tumour associated macrophages can have obvious differential effects on tumour growth (Zhu *et al.*, 2017). Understanding the exact ontogeny of endometriosis lesion resident macrophages is therefore an important step before the roles of these populations in disease can be deciphered.

The macrophage recruitment pathways active in endometriosis are currently unknown. We know for example that tumour associated macrophages in mouse models of breast cancer are recruited via CCL2 and that this recruitment process facilitates the growth of tumours (Qian *et al.*, 2011). Endometriosis lesions in human and mice are highly infiltrated by F4/80+ macrophages (Greaves *et al.*, 2014), however the mechanism of their recruitment into lesion tissue is poorly understood.

The phenotype of macrophages within endometriosis lesions has previously been defined as 'M2' like with few markers such as CD206 and Tie-2 being identified (Bacci *et al.*, 2009). However, we know in a tumour setting that multiple macrophage populations can exist which exhibit phenotypic heterogeneity, and these populations play differential roles in disease development (Quatromoni and Eruslanov, 2012). It is currently unknown whether multiple macrophage populations exist within endometriosis lesions. Current endometriosis research relies on the 'M1' and 'M2' dogma and has therefore largely failed to appreciate the complexity of macrophage phenotype and explore the possibility that multiple populations may exist.

Despite the number of growing investigations on the role of macrophages in endometriosis, data on macrophage origins, recruitment pathways and phenotypic heterogeneity in lesions is lacking. A more in-depth evaluation of lesion macrophages in light of current knowledge of the complexities of macrophage biology is required. Defining these aspects of lesion macrophage biology is a critical first step in the development of therapies which could potentially target lesion resident macrophage populations or prevent their influx into lesions to provide clinical benefit for women.

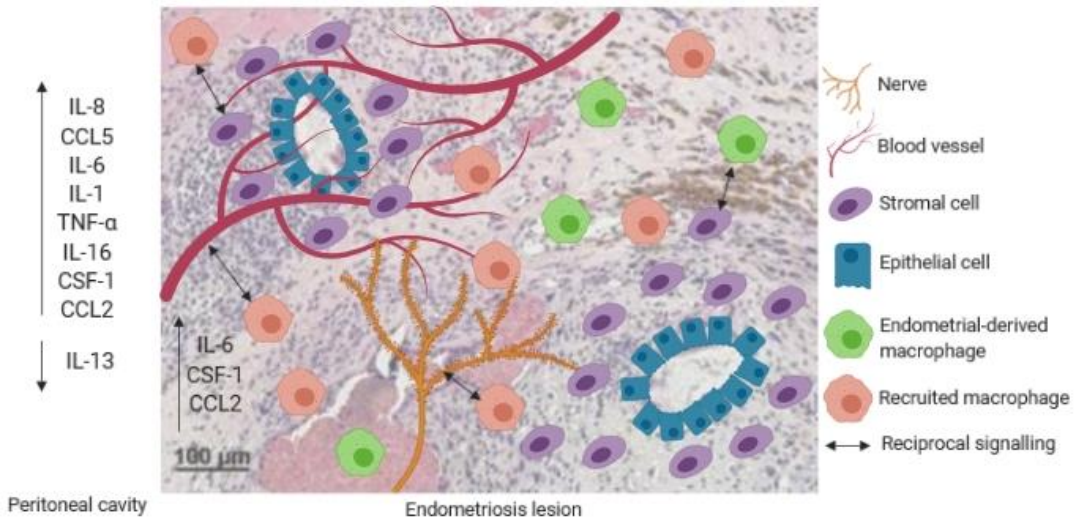


Figure 1.5. Endometriosis lesions are infiltrated by blood vessels, nerves and macrophages*. Lesion resident macrophages are derived from macrophages originating from the endometrium and recruited macrophages. Macrophages interact with blood vessels and nerves to stimulate their growth. Signalling also occurs between macrophages and stromal cells, which increases their clonal expansion and invasive properties. Women with endometriosis have increased peritoneal fluid levels of IL-8, CCL5, IL-6, IL-1, TNF-α, IL-16, CSF-1, CCL2, and a lower concentration of IL-13. CSF-1, CCL2 and IL-6 levels are also up-regulated in endometriosis lesions.*

1.8 Hypothesis and aims

I hypothesised that host-derived endometriosis lesion-resident macrophages have different origins and are recruited via CCR2, and that endometriosis-associated macrophages exhibit phenotypic heterogeneity.

My aims were to:

1. Determine the origin of macrophages in endometriosis lesions
2. Explore the role of CCR2-CCL2signalling in macrophage recruitment in endometriosis
3. Determine phenotypic heterogeneity of macrophages in endometriosis lesions

Chapter 2 – Materials and methods

2.1 Animals

2.1.1 General husbandry and ethics

All animal work was licensed and carried out in accordance with the UK Home Office Animal Experimentation (Scientific Procedures) Act 1986. This work was performed under my personal licence (PIL; I7F2EEADD). Categories A and B (modules 1-3) were acquired from the University's Accreditation Board, Cambridge 2014, and category C (module 4) was acquired from the Scottish Accreditation Board, Edinburgh 2016. All of the work was carried out under the project licence (PPL) of Dr Erin Greaves (70-8731). The work was performed by myself unless otherwise stated. Bioresearch and Veterinary Services provided general day-to-day animal husbandry in the University of Edinburgh animal facility Little France 2 (LF2), where all of the work in this thesis was carried out. The pathogens known to be present in LF2 are entamoeba, trictrichomonas and mouse norovirus. Where mice were imported into the unit, a week's acclimatisation period was allowed before any procedures were undertaken. Mice had access to food and water *ad libitum* and were kept at an ambient temperature and humidity of 21°C and 50% respectively. Light was provided 12 hours a day from 7am-7pm. Mice were typically housed in groups of 4-6 in clear, solid bottom cages. Clinical signs were monitored as well as weight of the mice. Where a 10% weight loss was recorded and/or adverse effects were observed, mice were humanely euthanized. The mouse model of endometriosis is classed as a moderate procedure, however actual severity was also recorded. Mice were culled by exposure to rising concentrations of CO_2 and cervical dislocation used as confirmation of death.

2.2 Mouse model of endometriosis

Estradiol-17β (Merck, Dorset, UK)

Sesame oil (Merck)

Progesterone (P4, Merck)

PBS tablets (Merck)

Estradiol valerate (Merck)

Non-surgical embryo transfer device (ParaTechs, Kentucky, USA)

Silastic® laboratory tubing (DowCorning™, Michigan, USA)

Scalpel

Our in-house mouse model of endometriosis provides an ideal platform for investigating the pathophysiology of endometriosis by mimicking the process of retrograde menstruation (Greaves *et al.*, 2014), one leading theory for the development of endometriosis (Sampson, 1927). Moreover, the model is ideal for our studies into the role of macrophages in endometriosis because the model is generated in mice with an intact immune system. The design also allows for the use of transgenic strains either in the donor or recipient mouse group, as long as donor and recipient strains are on the same genetic background.

The process of retrograde menstruation is simulated by inducing endometrial differentiation (decidualization) and breakdown in mice (Cousins *et al.*, 2014), allowing the transfer of 'menses'-like endometrial tissue into the peritoneal cavity of recipient mice which go on to develop lesions which phenocopy those seen in women with endometriosis (Greaves *et al.*, 2014). Specifically, donor mice were induced to undergo a menses-like event by manipulating hormonal exposure. On day 0 mice were ovariectomised and allowed to recover for 7 days, prior to receiving subcutaneous injections of 100ng estradiol-17β in 0.1ml sesame oil for 3 consecutive days from day 7 to 9. This mimics the estrogen dominated (proliferative) phase of the human menstrual cycle. This was then followed by a 3 day break and a further 3 days of 5ng estradiol-17β on days 13, 14 and 15. On day 13 mice also received a subcutaneous progesterone implant at the time of injection, made from Silastic® laboratory tubing. Days 13-15 mirror the progesterone dominated (secretory) phase of the menstrual cycle. A decidualization stimulus was given by introducing 20µl sesame oil into the lumen of the uterus via the cervix on day 15 using a non-surgical embryo transfer device. On day 19 (4 days after decidualization stimulus) the progesterone pellet was removed to induce progesterone withdrawal - a process vital for endometrial breakdown. Donor mice were culled 4 hours post withdrawal of

progesterone and the endometrial tissue was collected in the process of being shed from the decidualized horn: ‘menses’ endometrial tissue was recovered from the uteri of donor mice by opening each horn of the uterus longitudinally and scraping the endometrial tissue away with a scalpel. Approximately 40mg of tissue (equivalent to one uterine horn) was suspended in 0.2ml PBS, passed through an 18 gauge needle to slightly disperse the tissue and injected by two injections (each 0.1ml), either side of the umbilicus into the peritoneal cavity of recipient mice. Recipients had been previously ovariectomised and primed with a single subcutaneous injection of 500ng estradiol valerate (EV) 3 days prior to receiving tissue. Recipient mice received EV injections three times a week from tissue injection for the duration of the experiment. Lesions were allowed to develop over 1, 2 or 3 weeks after which the animals were sacrificed. Where appropriate naïve and/or sham (ovariectomy + intraperitoneal injection of PBS + EV) controls were included.

2.3 Transgenic mouse lines

2.3.1 C57 BL/6JOIaHsd

Wild-type C57BL/6JOIaHsd female mice were purchased from Harlan (Harlan Sprague Dawley Inc, Bicester, UK) at 8-12 weeks of age. This is an inbred, commonly used laboratory strain and was particularly useful in my studies utilizing the syngeneic mouse model of endometriosis because many transgenic lines are constructed on the C57BL/6 genetic background. All transgenic mice used were on a C57BL/6 genetic background unless otherwise stated.

2.3.2 ROSA26-rtTA:tetO-Cre:Csf1rflox/flox

Colony stimulating factor 1 receptor (Csf1r) conditional knock out (ROSA26-rtTA:tetO-Cre:Csf1rflox/flox) mice were used to study the role of endometrial macrophages in lesion development. This mouse strain has a conditional (floxed) knockout allele for Csf-1 receptor (Csf1r), reverse tetracycline-controlled transactivator (rtTA), and tetracycline-responsive promoter operated Cre (tetO-Cre). The Cre-Loxp system for targeted deletion of genes is described in *figure 2.1*. The floxed allele of Csf1r in these triple mutants was knocked out by Cre recombinase after treatment with the tetracycline analog doxycyclinehyclate (2μg/ml in 5%

sucrose water; Merck) causing Csf1r expressing macrophage populations to be depleted (Li *et al.*, 2006). Macrophage depletion was confirmed using flow cytometry.

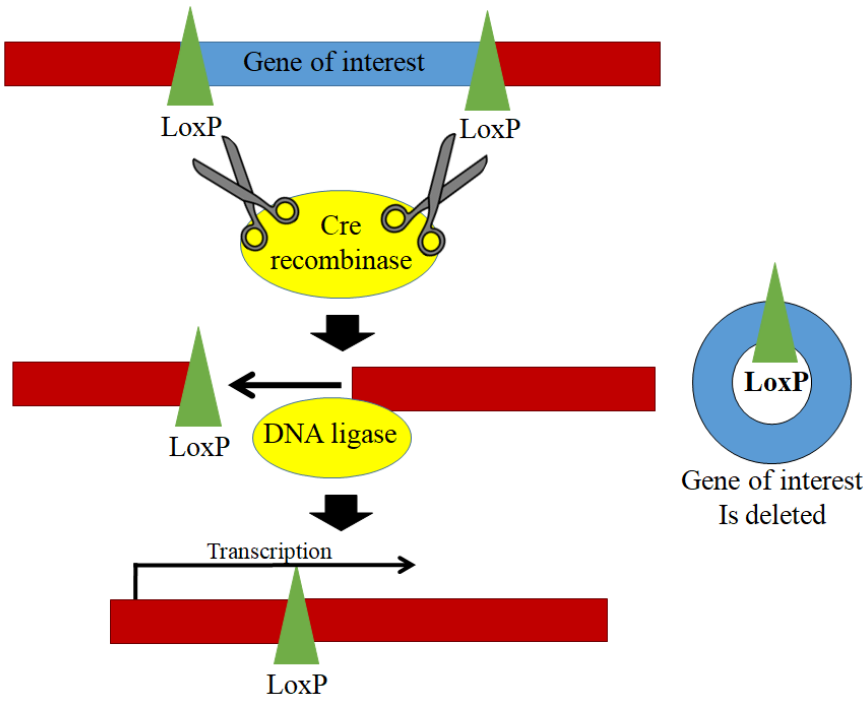


Figure 2.1. The Cre-Loxp system for selected depletion of genes. *Schematic of the Cre – LoxP system for targeted deletion of genes. This system makes it possible to delete selected genes in specific cell types in adult mice. Firstly, 34 base pair LoxP sites are inserted either side of a gene of interest. These LoxP sequences contain specific 13 base pair regions that the Cre recombinase enzyme, isolated from bacteriophage P1, can recognize. The Cre recombinase enzyme is able to catalyse DNA recombination. In the presence of Cre, a recombination event occurs between the two LoxP sites, causing the gene of interest to be cut from the DNA. DNA ligase then re-joins the DNA such that transcription can occur, however with negation of the selected gene.*

2.3.3 B6.Cg-Tg(Csf1r-EGFP)1Hume/J

B6.Cg-Tg(Csf1r-EGFP)1Hume/J (hereby referred to as 'MacGreen') mice were used for fate-mapping experiments aimed at tracking macrophage infiltration into endometriosis lesions. MacGreen mice express enhanced green fluorescent protein (EGFP) under control of the c-fms gene, encoding for CSF-1 receptor (CSF-1r), thus macrophage populations expressing CSF-1 receptor can be identified by EGFP expression (Sasmono *et al.*, 2003).

2.3.4 B6.129S4-*Ccr2*^{tm1Ifc}/J

B6.129S4-*Ccr2*^{tm1Ifc}/J (C-C chemokine receptor type 2 (Ccr2) -/-) mice were used to study the role of monocytes and monocyte-derived macrophages in endometriosis lesion development. These were gifted from Steve Jenkins (Centre for Inflammation Research, UoE) and bred in house. Ccr2-/- mice have a homozygous mutation in the Ccr2 gene, which was achieved by replacing the entire coding region except the first 39 nucleotides and 5' untranslated region (Boring *et al.*, 1997). These mice have reduced monocytes, monocyte-derived macrophages and small peritoneal macrophages in the peritoneal cavity and a low number of circulating Ly6C^{hi} monocytes due to an inability for monocytes to extravasate from the bone marrow and from blood vessels, processes regulated by CCR2 (Boring *et al.*, 1997).

2.3.5 B6.129S4-*Ccl2*^{tm1Rol}/J

B6.129S4-*Ccl2*^{tm1Rol}/J (Chemokine (c-c motif) ligand 2 (Ccl2) knock out) mice were used to study the role of CCL2 in the development of endometriosis lesions in mice. Ccl2 knockout mice contain a mutation in the *SCYA2* gene encoding the CCL2 ligand. Ccl2 KO mice have normal peritoneal macrophage numbers but reduced recruitment of monocytes and monocyte-derived macrophages into the peritoneal cavity under inflammatory conditions (Lu *et al.*, 2002).

2.3.6 FVB-Tg(CAG-luc,-GFP)L2G85Chco/J

FVB-Tg(CAG-luc,-GFP)L2G85Chco/J mice were purchased from Jackson Laboratories (Stock no: 008450|L2G85; hereby referred to as CAG-luc) on an FVB background. CAG-luc mice were bred in-house and used to monitor the establishment of endometriosis lesions over time using bioluminescent imaging (described in 2.4). CAG-luc mice exhibit global expression of firefly luciferase under control of the CAG promoter. Upon subcutaneous injection of luciferin (150mg/kg), luciferin is oxidised in the presence of luciferase and produces energy in the form of light (Cao *et al.*, 2004). Donor CAG-luc mice were used to generate endometrial tissue, which was injected into the peritoneal cavity of recipient wild type mice such that lesions exhibited bioluminescence under stimulation of luciferin.

2.4 *In vivo* bioluminescent imaging

PhotonIMAGER™ (Biospace Lab, Nesles la Vallée, France)

D-luciferin potassium salt (ThermoFisherScientfic)

M3 vision software (Biospace Lab)

Bioluminescent lesions were visualized *in vivo* using a PhotonIMAGER™. Endometriosis mice were injected with 150mg/kg0.1ml luciferin subcutaneously before imaging, such that only vascularized endometriosis lesions were visualized. Mice were anaesthetized and imaged for 7 minutes on both their front and back. Fluorescent images were visualized using M3 vision software.

2.5 Genotyping

2.5.1 Ear notch digestion and DNA quantification

50µl 25mM NaOH 0.2mM EDTA made up in dH_20

50mM Tris-HCL made up in dH_20

Techne®DRI.block®heater (Merck)

Nanodrop One (ThermoScientific, Madison, USA)

Ear notches were stored at -20°C. On the day of genotyping, ear notches were thawed and DNA extraction achieved by incubation of the notches with 50µl 25mM NaOH 0.2mM EDTA made up in dH_20 at 95°C on a Techne®DRI.block®heater for 20 minutes. After incubation, 50µl 50mM Tris-HCL made up in dH_20 was added to the ear notches and briefly vortexed. DNA from each ear notch was quantified by spectrophotomic analysis using a Nanodrop One and standardized to 100ng/µl by diluting in nucleic acid free dH_2O.

2.5.2 Polymerase chain reaction (PCR)

MyTaq™ Red Mix master mix (Bioline, London, UK)

Forward and reverse primers (*Table 2.1*)

BioerLifeECO™ thermal cycler (Alpha Laboratories, Eastleigh, UK)

A PCR reaction (final volume 25µl) was prepared to amplify the desired gene product. 12.5µl MyTaq™ Red MixPCR master mix, 10.5µl water, 0.5µl forward and reverse primers (at 20µM) and 1µl DNA were added to the reaction. The reaction was briefly vortexed and placed into a BioerLifeECO™ thermal cycler. The thermal cycling conditions were: initial denaturation at 96°C for 2 minutes, followed by 35 cycle of denaturation at 95°C for 20 seconds, annealing at 57°C for 20 seconds, extension at 72°C for 30 seconds. The final extension was at 72°C for 7 minutes.

Gene	Forward primer	Reverse primer	Neo Flox	Source
CCR2	CCACAGAATCAAAGGAAATGG	TGCCCACAAAACCAAAGATGA A	GCATCGCCTTCTATCGCCTTCTTGA	The Jackson Laboratory
GFP	AAGTTCATCTGCACCACCG	TGCTCAGGTAGTGGTTGTCG	/	Eurofins Scientific

Table 2.1. Table of primers used for genotyping

2.5.3 Gel electrophoresis and visualisation of DNA

Agarose (Bioline, London, UK)

10 x TAE buffer (48.4g Tris Base, 11.4ml acetic acid, 3.7g EDTA made up in dH_20)

100 base pair DNA ladder (500µg/ml) (New England Biolabs, Hitchin, UK)

U:Genius^3 (Syngene International Limited, Cambridge, UK)

PCR products were subject to gel electrophoresis on a 2% agarose gel (with 10µl gel red) made with 10x TAE buffer diluted 1:10 in dH_20. The products were separated at 150 mVa for 40 minutes. A 100 base pair DNA ladder was added either side of the samples for visualization of the appropriate DNA band. The gel was then imaged using U:Genius^3.

2.6 Flow cytometry and fluorescence activated cell sorting (FACS)

2.6.1 Peritoneal lavage fluid collection

Dulbecco's modified eagle medium (DMEM) (Gibco, Inninchan, Scotland)

26 gauge needle

10 ml syringe

15ml Falcon tube

Peritoneal lavage cells were collected by injecting 7ml ice cold DMEM into the peritoneal cavity of mice followed by gentle massage and retrieval into a 15ml Falcon tube. The samples were then centrifuged at 500 relative centrifugal force (rcf) for 7 minutes and the supernatant discarded. The pellet was then taken forward for red blood cell lysis.

2.6.2. Tissue collection and digestion

DMEM

Liberase DL (Roche, Welwyn Garden City, UK)

Liberase TL (Roche)

DNAse (Merck)

PBS tablets (Merck)

Flow buffer (PBS + 2% BSA)

15ml Falcon tube

50ml tubes

Scalpel

Water bath set to 37°C (Scientific Laboratory Supplies, Coatbridge, UK)

100μM filters (Falcon, USA)

Flow cytometry was performed on both decidualised endometrial tissue and endometriosis lesions from mice. For decidualised endometrial tissue, mice were induced to undergo a 'menses'-like event as previously described (Cousins *et al.*, 2014). On day 19, P4 pellets were removed and 4 hours later, the mice were culled. The uterine horn was opened longitudinally and the decidualised endometrial tissue scraped away using a scalpel, and placed in 2ml ice-cold DMEM. For endometriosis

lesions, lesions were induced to form in mice as previously described (Greaves *et al.*, 2014). 2 weeks post injection of tissue, lesions were dissected, pooled from each mouse and placed in 2ml ice-cold DMEM. Prior to digestion, endometrium or lesions were cut into small pieces using a scalpel. The tissue was then transferred to a 15ml tube and the volume adjusted to 4ml with serum free PBS. 40µl 26U/ml Liberase DL, 80µl 13U/ml Liberase TL and 40µl 15mg/ml DNAse enzymes were added. The tissue was incubated for 45 minutes in a 37°C water bath, and vortexed every 5 minutes. For single cell RNA sequencing samples, the samples were not vortexed throughout the protocol but pipetted up and down to mix, so as to minimise damage to cells. To stop the digestion 10 ml flow buffer (PBS + 2% BSA) was added, and the samples were filtered through 100µM filters into 50ml tubes. The volume was adjusted to 20ml with flow buffer and the samples centrifuged at 500g for 7 minutes.

2.6.3 Red blood cell lysis and blocking of excess binding sites

10 X RBC lysis buffer (Biolegend, London, UK)

Anti CD16/CD32 blocking antibody (BD Pharminogen, Wokingham, UK)

Flow buffer (PBS + 2% BSA)

After centrifugation, peritoneal lavage, endometrial or lesion resident cells underwent red blood cell lysis by incubation with 3ml 10 X RBC lysis buffer diluted 1:10 in dH_20 for 10 minutes. Excess binding sites on cells were then blocked for 30 minutes at 4°C with 2µl (1:500) of anti CD16/CD32 blocking antibody added to the cells in 1ml flow buffer. The cells were vortexed every 10 minutes.

2.6.4 Fluorescent staining of cells

Luna™ dual fluorescence cell counter (Labtech, Sussex, UK)

Fluorochrome conjugated antibodies (*Table 2.2*)

Brilliant™ Stain Buffer (BD Horizon, Wokingham, UK)

4′,6-diamidino-2-phenylindole(DAPI) (Merck)

123count eBeads™ (eBioscience, Inninchan, Scotland)

Cells were counted using a Luna™ dual fluorescence cell counter and adjusted to 1 million cells/ml before staining with a cocktail of antibodies selected based on spectral overlap properties (*Figure 2.2*). See Table 2 for a list of all antibodies used. Where more than one Brilliant™ Violet (BV) fluorochrome was used, 50µl Brilliant™ Stain Buffer was added to the sample to negate non-specific interactions between the BV polymer dyes, which can affect spread of the data and introduce artefact populations. Samples were then stained with 1µl 1:400 DAPI (original stock 5mg/µl, final concentration 0.0125mg/µl) diluted in dH_20 before analysis for live/dead differentiation. 100µl of 123count eBeads™ were added to each sample for accurate determination of cell counts (cells/µl) during analysis.

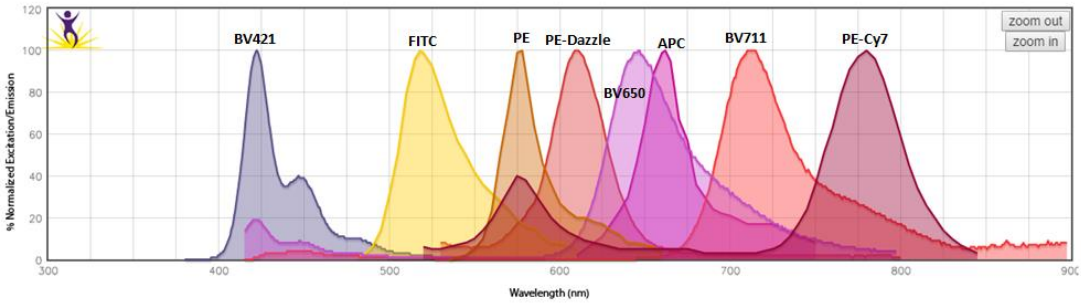


Figure 2.2. Spectral analysis used to design flow cytometry panels. *Spectral analyser used for designing flow cytometry antibody panels. The peaks shown represent the emission spectrum of each fluorochrome. Each panel is designed such that there is minimal overlap between the peaks. The more overlap the more difficult it is to perform compensation and efficiently determine the fluorescence of a given cell population. Figure adapted from www.biolegend.com/spectralanalyzer.*

Marker	Fluorochrome	Dilution	Clone
CD11b	PE-Dazzle	1 in 800	M1/70
CD11c	APC-Cy7	1 in 200	N418
CSF1R	APC	1 in 200	AFS98
CSF1R	PE	1 in 200	AFS98
F4/80	PECy7	1 in 200	BM8
F4/80	PERCP-Cy5.5	1 in 200	BM8
F4/80	AF488	1in 200	BM8
Ly6C	BV711	1 in 400	HK1.4
Ly6G	BV650	1 in 200	1A8
MHC II	AF700	1 in 400	M5/114.15.2
CD45.2	PERCP-Cy5	1 in 200	104
CD45.2	APC	1 in 200	104
CD3	FITC	1 in 200	17A2
CD3	APC	1 in 200	17A2
B220	FITC	1 in 200	RA3-6B2
CD19	FITC	1 in 200	1D3-CD19
CD19	APC	1 in 200	1D3-CD19
CD335	FITC	1 in 200	29A1.4
CD49B	APC	1 in 200	DX5
CX3CR1	Pe-Cy7	1 in 200	SA011F11
CCR2	PE	1 in 200	SA203G11
CD86	APC-Cy7	1 in 200	GL-1
CD200	APC	1 in 200	OX-90
CCR5	PE	1 in 200	HM-CCR5

Table 2.2. Table of antibodies used for flow cytometry and FACS

2.6.5 Flow cytometry

OneCompeBeads (Thermo Fisher Scientific, Madison, USA)

6 laser LSR Fortessa (Becton Dickinson, New Jersey)

BD FACS DIVA™ software (BD Biosciences, San Jose, USA)

123count eBeads™ (eBioscience, Inninchan, Scotland)

Stained samples were analysed on a 6 laser LSR Fortessa in the QMRI flow cytometry facility. Each experiment was compensated using single stained OneComp beads for each fluorochrome in the cocktail using the automatic compensation tool on BD FACS DIVA™ software and a fully stained sample to validate compensation parameters. Where positive and negative cell populations were not clearly distinguishable, fluorescence minus one was performed for delineation of populations. For fluorescence minus one, every antibody apart from the antibody against the un-distinguishable population in question was added to the sample. This allowed identification of cells that were truly positive for the antibody and auto fluorescent populations identified, allowing accurate gating of positively stained populations.

2.6.6 Data analysis

Data analysis was performed using FlowJo® 10.2 software (Tree Star Inc, Ashland, USA). Unstained samples were used for visualization of positively stained populations and fluorescence minus one controls included as appropriate when gating was not clear. Cells which stained positively for DAPI were negated from analysis as only dead cells incorporate DAPI into their DNA (*figure 2.3A*). Doublets were excluded from the analysis by gating out cells with a high side scatter (height) compared to side scatter (area) (*figure 2.3B*). Eosinophils, B cells, T cells and NK cells were removed by gating out cells positive for siglec F, CD19, CD3 and CD335 antibodies respectively (lineage gate) which were all conjugated to the same fluorochrome (FITC or APC) (*figure 2.3C*). Cell populations were then enumerated by positive gating on desired populations as well as the 123count eBeads™ added to the sample (*figure 2.3D*). Absolute cell counts were determined using the equation: Cells/μl count = (cell count ÷ eBead count) x eBead batch concentration.

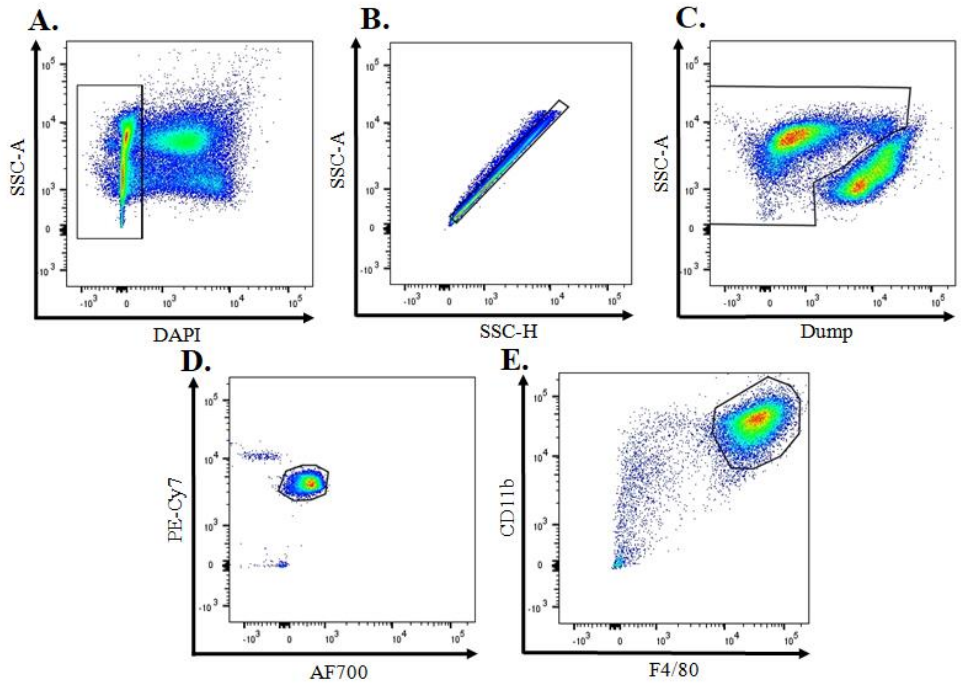


Figure 2.3. Flow cytometry gating strategy. A*: Dead cells were excluded from the analysis by gating around cells which were negative for DAPI.* ***B****: Doublets (cells stuck together) were excluded from the analysis by excluding cells with a high SSC-H to SSC-A ratio.* ***C****: Eosinophils, B cells, T cells and NK cells were negated from the analysis using the lineage gate.* ***D****: 123count eBeads were visualised using fluorescence in the PE-Cy7 and AF700 channels and gated for accurate enumeration of cells/µl of a given population.* ***E****: Example of gating on a CD11b+ F4/80+ macrophage population.*

2.6.7 Fluorescence-activated cell sorting (FACS)

FACS Fusion (Becton Dickinson, New Jersey)

BD FACS DIVA^{TM} software (BD Biosciences, San Jose, USA)

Red blood cell lysis, Fc blocking and fluorescent staining were performed as previously described and samples sorted into pure cell populations based on cell surface marker expression using a FACS Fusion (Becton Dickinson, New Jersey). Cell sorting is achieved using a stream of sheath fluid which separates out cells into a single cell suspension, and an electrostatic separation technique whereby cells are given a charge and separated based on that charge. Cells first enter the system into the stream of sheath fluid, which causes separation of single cells into a stream. At the interrogation point, lasers hit the cells and the fluorescence, reflective of cell surface marker expression, is determined, much the same as with flow cytometry.

Using BD FACS DIVA^{TM} software the cells of interest are selected. A pizoelectrical crystal creates oscillations at the end of the stream of cells, causing each cell at the end of the stream to be isolated in a droplet of fluid. This droplet can be given either a negative or positive charge, and is collected at charged magnetic collection columns, thus allowing collection of a specified cell of interest based on cell surface marker expression. Dead cells and doublets were excluded from analysis as previously described. BD FACS DIVA^{TM} software was used for visualization and gating of cell populations for sorting.

2.7 Histology and immunohistochemistry

2.7.1 Tissue fixation and processing

Xylene

4% neutral buffered formaldehyde (NBF) in PBS

Leica TP 1050 processor (Leica Biosystems, Newcastle upon Tyne, UK)

Microm HM 315 microtome (Leica Biosystems, Wetzlar, Germany)

Positively charged glass slides (CellPath, Newtown, Wales)

Ethanol solutions in water (95-70% ethanol)

At tissue collection, tissues were placed in 4% neutral buffered formaldehyde (NBF) for 24 hours and stored in 70% ethanol thereafter. Tissue was embedded in wax blocks by the Shared University Research Facility (SURF) histology staff using the Leica TP 1050 processor. For sectioning, wax blocks were chilled on ice before being cut into 5µm sections on a hand operated MicromHM 315 microtome. The sections were floated on warm water before being placed on a positively charged glass slides and incubated for 24 hours at 55°C. On the day of staining, slides were first dewaxed in xylene for 10 minutes. Slides were then re-hydrated by incubation in gradually decreasing concentrations of ethanol for 20 seconds each (95-70%).

2.7.2 Haematoxylin and eosin staining of lesions

Harris's haematoxylin (Triangle Biomedical Sciences)

Acid alcohol (1% HCL in 70% v/v ethanol)

Scott's tap water (20g $MgSO_4$, 3.5g sodium bicarbonate in 1 litre distilled H_20)

Eosin (Triangle Biomedical Sciences)

Ethanol solutions in water (95-70% ethanol)

Xylene Glass coverslips (VWR, Poole, UK)

Pertex® (Cell Path, Hemmel Hempstead, UK)

After dewaxing and re-hydration, samples were incubated for 5 minutes in Harris's haematoxylin for nuclear staining. Sections were then washed in tap water and placed in 1% acid alcohol for 10 seconds. After being washed in water, samples were then incubated in Scott's tap water for 30 seconds. To add a cytoplasmic stain, samples were incubated in eosin for 30 seconds. Dehydration was then achieved by incubation in gradually increasing concentrations of ethanol (70-95%) and the samples cleared by incubation in xylene for 10 minutes. The slides were then mounted with glass coverslips using Pertex®.

2.7.3 Cytokeratin and vimentin immunohistochemistry

Tris-buffered saline (6.05g Tris, 8.76g NaCl in 1L H_20, PH 7.5)

0.01M citrate buffer (25ml 0.1M citrate in 225ml H_20)

3% H_20_2 (Merck) in methanol

Streptavidin and Biotin block (Vector, Peterborough, UK)

Blocking serum (1:4 NGS:PBS, 5% BSA)

Primary antibodies (*table 4*)

Goat anti-mouse biotinylated secondary antibody (Merck)

ImmPRESS® horse radish peroxidase (HRP)-conjugated anti-rabbit secondary antibody (Vector, Peterborough, UK)

Streptavidin horseradish peroxidase (Santa Cruz Biotechnology, Heidelberg, Germany)

3,3-diaminobezidine (DAB) (Vector, Peterborough, UK)

Harris's haematoxylin (Triangle Biomedical Sciences)

Instant Pot®(IP-LUX50) pressure cooker

Pertex®

Immunodetection of cytokeratin and vimentin was performed on endometriosis lesions from mice for identification of epithelial and stromal cells respectively. After dewaxing and re-hydration the slides were washed in tap water and Tris-buffered saline (6.05g Tris, 8.76g NaCl in 1L H_20, PH 7.5). Antigen retrieval was performed by immersing slides in 0.01M citrate buffer (25ml 0.1M citrate in 225ml H_20) and exposing them to pressurised heating for 5 minutes in an Instant Pot® pressure cooker. After antigen retrieval slides were allowed to cool for 30 minutes before being washed in tap water. To block endogenous peroxidase, slides were incubated in 3% H_20_2in methanol for 15 minutes and washed in Tris-buffered saline (TBS) for 2 x 5 minutes. A streptavidin block was applied to the sections for 15 minutes and slides washed 2 x 5 minutes in TBS. A biotin block was then applied for 15 minutes and slides washed in TBS 2x for 5 minutes. Slides were then incubated for 30 minutes with blocking serum (1:4 normal goat serum (NGS): PBS, 5% BSA). After blocking, either cytokeratin (1:2000) or vimentin (1:600) primary antibodies were incubated with the sections overnight, diluted in blocking serum. Controls where primary antibody was omitted were incubated in blocking serum only overnight. After overnight incubation slides were washed 2 x 5 minutes in TBS before adding secondary antibodies. For the cytokeratin stain a goat anti-mouse biotinylated secondary antibody was diluted 1:500 in blocking serum and added to the slides. For the vimentin stain one drop of ImmPRESS® HRP conjugated anti-rabbit antibody was added to the slides. These secondary antibodies were incubated with the sections for 30 minutes. Slides were then washed 2x 5 minutes in TBS. Streptavidin horseradish peroxidase (HRP) was then diluted 1:1000 in TBS and added to the

sections being stained for cytokeratin for 30 minutes. At this stage vimentin slides were left in TBS as they had already received a HRP-conjugated secondary antibody (1 step detection as opposed to 2 step for cytokeratin). All of the slides were then visualized by incubation with 3,3-diaminobezidine (DAB) for 10 minutes before stopping the reaction in water. Sections were then counterstained with haematoxylin for 5 minutes, dehydrated and mounted using Pertex®.

2.7.4 Immunohistochemical stain imaging

PROVIS microscope (Zolympus Optical, London, UK)

AxiocamHRc (Zeiss) camera

ZEN 2.3 pro software (Carl Zeiss, Hertfordshire, UK)

Cytokeratin, vimentin and haematoxylin and eosin stains were imaged using a PROVIS microscope. An AxiocamHRc (Zeiss) camera was used to capture images using ZEN 2.3 pro software.

2.7.5 Immunofluorescence

0.01M citrate buffer (25ml 0.1M citrate in 225ml H_20)

Instant Pot®(IP-LUX50) pressure cooker

1mg/ml trypsin from porcine pancreas (Merck)

3% H_20_2 in methanol

Tris-buffered saline (6.05g Tris, 8.76g NaCl in 1L H_20, PH 7.5)

Blocking serum (1:4 normal goat serum (NGS): PBS, 5% BSA)

Primary antibodies (*table 4*)

ImmPRESS® HRP-conjugated anti rabbit/rat secondary antibodies (Vector)

Tyramide Signal Amplification kit (PerkinElmer, Buckinghamshire, UK)

DAPI (Merck)

PermaFluor™ (Thermo Fisher Scientific)

Dual immunofluorescence was used to identify monocyte and macrophages present in mouse endometriosis lesions. For dual immunofluorescence, sections were dewaxed and re-hydrated prior to antigen retrieval by either incubation in 0.01M citrate buffer and heating in an Instant Pot® pressure cooker for 5 minutes (GATA6, Ly6C and GFP) or incubation for 10 minutes at room temperature in 1mg/ml trypsin from porcine pancreas made up in dH_20 (F4/80). Endogenous peroxidases were blocked using 3% H202 in methanol and slides washed for 2 x 5 minutes in TBS. Slides were then immersed in blocking serum (1:4 normal goat serum (NGS): PBS, 5% BSA) for 30 minutes. After blocking excess binding sites slides were incubated in primary antibodies F4/80 (1:600), GATA6 (1:3000), GFP (1:2000) or Ly6C (Abcam,1:100) diluted in blocking serum overnight. Controls with no primary antibody were incubated in blocking serum overnight. imPRESS® HRP-conjugated secondary antibodies were then added to the sections (anti-rat for F4/80 and Ly6C, anti-rabbit for GATA6 and anti-mouse for GFP) for 30 minutes and visualized using Tyramide Signal Amplification, which was added to the slides for 7 minutes (tyramide reagent diluted 1:50 in diluent). At this stage the slides were then stained for a secondary antibody by repeating the protocol as previously described. In brief, antigen retrieval, blocking of endogenous peroxidases and blocking of excess binding sites was achieved. The second primary antibodies were then added to the slides and incubated overnight. Slides were incubated with secondary antibodies and visualized using Tyramide Signal Amplification, using a different fluorochrome for detection of the second antibody. The slides were then washed 2 x 5 minutes in TBS and incubated with the nuclear stain DAPI diluted 1:500 in TBS for 10 minutes. Slides were finally washed in TBS for 5 minutes and mounted with glass coverslips using PermaFluor™.

Antibody	Source	Catalog number	Description of target	Species raised	Concentration of primary	Secondary used
Cytokeratin	Merck	C2562	Epithelial cells	Mouse	1:2000	Goat anti-mouse biotinylated antibody (diluted 1:500)
Vimentin	Cell Signalling Technology inc	5741S	Stromal cells	Rabbit	1:600	ImmPRESS® HRP-conjugated anti-rabbit antibody
F4/80	eBioscience	14-4801	Macrophages	Rat	1:600	ImmPRESS® HRP-conjugated anti-rat antibody
GATA6	Cell Signalling Technology	5851S	Large peritoneal macrophages	Rabbit	1:3000	ImmPRESS® HRP-conjugated anti-rabbit antibody
Ly6C	Abcam	ab15627	Monocytes and monocyte-derived macrophages	Rat	1:100	ImmPRESS® HRP-conjugated anti-rat antibody
GFP	Abcam	ab6556	Macrophages expressing Csf1r-EGFP	Mouse	1:2000	ImmPRESS® HRP-conjugated anti-mouse antibody

Table 2.3. Table of primary and secondary antibodies used in immunohistochemistry and immunofluorescence.

2.7.6 Imaging of immunofluorescent stains

Fluorescent images were captured using a Zeiss LSM 780 confocal microscope (Carl Zeiss, Hertfordshire, UK).

2.7.8 Image analysis

2.7.8.1 Fiji analysis

For cell counting of F4/80 GATA6 dual immunofluorescent stains, 4 random images at x63 objective were taken from each lesion and images quantified using Fiji plugin 'Cell Counter'. Total nuclei were counted, as well as cells positive for respective markers. Values were expressed as % of total DAPI+ cells.

2.7.8.2 Definiens architect XD™ analysis

For F4/80 Ly6C dual immunofluorescent stains and single GFP stains, image analysis was carried out by Dr Daniel Soong using Definiens Architect XD™ software. Tissue sections were first classified as either lesion, haemorrhage, fat or peritoneum. Areas of haemorrhage and fat were removed from the analysis and lesion and peritoneal tissue taken forward for cell counting. The total number of cells were counted as well as the number of cells positive for respective antibodies on the sections. Areas of lesion and peritoneum were counted separately.

2.8 Statistical analysis

Statistical analysis was carried out in GraphPad Prism 7.02. Data was first analysed for normality using an Anderson Darling normality test. If data were normal, either an ANOVA with a Tukey's post-hoc test (more than 2 samples) or a t-test (2 samples) was performed. If data was not normal, non-parametric statistical tests were used, either Kruskal-Wallis with a Dunn's post hoc test (more than 2 samples) or a Mann-Whitney U test (2 samples). In the case where a study had a small n number (e.g. 3 mice), non-parametric tests were utilised and data presented as median and range in order to reduce the likelihood of type 2 errors (false negatives). Otherwise, data was presented as mean and standard error of the mean (SEM). Statistical significance was reported at $p=<0.05$.

2.9 Single cell RNA sequencing

2.9.1 Barcoding of samples using the chromium controller

PBS + 2% FBS

TC20™ automated cell counter (Bio-Rad Laboratories, California, USA)

Chromium controller™(10x genomics, Leiden, Holland)

Chromium™ single cell A chip (10x genomics)

10x™ gasket (10x genomics)

Single Cell 3’ Gel Bead Strip (10x genomics)

C1000 touch™thermal cycler (Bio-Rad Laboratories, California, USA)

RT reagent mix (10x genomics)

RT primer (10x genomics)

Addative A (10x genomics)

RT enzyme mix (10x genomics)

Nuclease free water (Thermo Fisher Scientific)

Partitioning oil (10x genomics)

200,000 CD45+ cells were sorted into 1ml PBS + 2% FBS using a FACS Fusion. Cells were then centrifuged at 500g for 5 minutes and re-suspended in 1ml PBS + 2% FBS and counted using a TC20™ automated cell counter**.** Cell number and viability were assessed and samples with a viability over 70% were taken forward for barcoding. Cells were centrifuged again at 500rcf for 5 minutes and re-suspended in 50µl PBS + 2% FBS. Cells were barcoded using a Chromium Controller™. Barcoding refers to the process which tags all of the RNA in each cell, giving each cell a unique oligonucleotide identifier which can be retrieved during analysis, thus enabling gene expression analysis at a single cell resolution. The 10x™ GemCode™ technology uses a pool of ~750,000 barcodes. Each cell is partitioned into a nanoliter-scale Gel Bead-In Emulsion (GEM) with a single barcode. Cells are introduced to the system at a limiting dilution, such that 90-99% of GEMs contain no cell. This ensures that only a single cell is incorporated into each GEM and thus only a single cell will have the same barcode. After the RNA of each cell is tagged by a barcode, the GEMs are lysed and primers containing a 16 nucleotide 10x barcode, a 10 nucleotide Unique Molecular Identifier (UMI, tags each sample), an Illumina®R1 sequence (primer required for sequencing, referred to as read 1) and a poly-dT primer sequence are mixed with the tagged RNA and Master Mix.

This barcoding process was achieved first by creating a reaction mix by adding 50µl RT reagent mix, 3.8µl RT primer, 2.4µl additive A and 10µl RT enzyme mix added to a PCR tube on ice. 25µl nuclease free water was added to the master mix, as well as 7µl cells. This was gently mixed and 90µl carefully added into row 1 on the single cell A chip. In row 2, 40µl Single Cell 3' Gel Beads (incorporated into the GEMs) was slowly added, being careful not to introduce air bubbles. 270µl partitioning oil was then added in two lots of 135µl into row 3. This is required to produce the oil droplets which form the GEMs. A 10xgasket was then used to cover the chip and the chip loaded onto the Chromium Controller™, which was run on a set protocol for 6 minutes. After running on the Chromium Controller™, samples were incubated in a thermal cycler in order to generate cDNA. The samples were taken through the following protocol: 53°C for 45 minutes, 85°C for 5 minutes, held at 4°C thereafter.

2.9.2 Library preparation

Library preparation refers to the process of preparing the samples for Illumina® bridge amplification sequencing. This was a 1.5 day process which cleaned the samples to get rid of reagents and primers, amplified cDNA to generate sufficient product, size selected to optimize the cDNA amplicon size and added the primers required for Illumina®sequencing.

2.9.2.1 Post GEM-reverse transcription clean up and cDNA amplification

C1000 touch™ thermal cycler (Bio-Rad, California, USA)

DynaBeads® MyOne™ Silane Beads (Thermo Fisher Scientific)

10x™ Magnetic Separator (10x genomics)

80% ethanol (Merck)

Nuclease free water (Thermo Fisher Scientific)

Elution solution (10x genomics)

cDNA amplification reaction mix (10x genomics)

Clean-up was performed to remove leftover primers and biochemical reagents from the post GEM reaction mixture, used to generate cDNA. In order to remove these reagents, DynaBeads® MyOne™ Silane Beads (hereby referred to as DynaBeads®) were used. The cDNA binds to the beads, allowing separation of the cDNA from leftover reagents and primers. The samples were mixed with 200µl DynaBeads® for 5 minutes, after which they were placed on a 10X™Magnetic Separator until the supernatants became clear, indicating that the beads had accumulated at the magnet. This allowed the supernatants, containing excess reagents and primers, to be discarded, leaving only the cDNA left on the beads. 150µl freshly prepared 80% ethanol in nuclease free water was then used to wash the beads twice for 3 seconds. 35.5µl elution solution was then added to the beads and the tubes placed onto the 10X™ Magnetic Separator for 1 minute. The supernatants now contained the purified cDNA and were taken forward for PCR. PCR was performed in order to generate

sufficient cDNA for library construction. cDNA was amplified by incubation with 65µl cDNA amplification reaction mix provided by 10x and the following protocol run on a thermal cycler: 98°C for 3.15 minutes, 67°C for 20 seconds, 72°C for 1 minute (repeated for 10 cycles), then 72°C for 1 minute and held at 4°C thereafter.

2.9.2.2 Post cDNA amplification reaction cleanup

Qubit 3 fluorometer bioanalyzer (Invitrogen, California, USA)

SPRIselect Reagent Kit (Beckman Coulter, California, USA)

10X™ Magnetic Separator (10x genomics)

80% ethanol (Merck)

Nuclease free water (Thermo Fisher Scientific)

EB Buffer (Qiagen, Hilden, Germany)

This stage of the protocol removed any leftover reagents from the cDNA amplification reaction mix and ensured that only the purified cDNA product was taken forward through the protocol. The same concept was utilised as in section 2.9.2.1 however instead of DynaBeads®, SPRIselect Reagent Kit (SPRI beads) was used, another type of magnetic beads. 60µl SPRI beads were added to the samples, incubated for 5 minutes and then placed on the 10X™ Magnetic Separator until the solutions were clear. The supernatants were discarded and the beads washed with 200µl 80% ethanol in nuclease free water for 30 seconds twice. The ethanol was removed and the tubes, still in the 10X™ Magnetic Separator, were incubated at room temperature for 2 minutes. The samples were taken off the 10x™ Magnetic Separator and 40.5µl EB buffer added to the samples and incubated for 2 minutes at room temperature. Samples were then placed back on the 10x™ Magnetic Separator until the solutions were clear, and 40µl supernatant containing the purified cDNA taken forward through the protocol. At this point the amount of cDNA was enumerated using a Qubit 3 fluorometric bioanalyzer to ensure a sufficient cDNA yield (>30ng/µl is optimal).

2.9.2.3 Fragmentation, end repair and A-tailing

C1000 touch™ thermal cycler (Bio-Rad, California, USA)

Fragmentation buffer (10x genomics)

Fragmentation enzyme blend (10x genomics)

SPRIselect Reagent Kit (Beckman Coulter, California, USA)

10x™ Magnetic Separator (10x genomics)

80% ethanol (Merck)

Nuclease free water (Thermo Fisher Scientific)

EB Buffer (Qiagen, Hilden, Germany)

This process selects for cDNA of specific sizes in order to optimize the cDNA amplicon size. This gets rid of cDNA which is too small or too large, and thus is called double sided size selection. This process assumes that cDNA of interest will be within a specific size range. cDNA underwent fragmentation, end repair (blunts the fragmented DNA) and A-tailing (adds an adenosine to the 3' end of the DNA). This was achieved by adding 5µl fragmentation buffer, 10µl fragmentation enzyme blend and 35µl purified cDNA to a PCR tube. This mix was transferred to a pre-cooled thermal cycler and underwent the following cycle: 32°C for 5 minutes (fragmentation stage), 65°C for 30 minutes (end repair and A-tailing) and held at 4°C thereafter. After the reaction, the samples were taken through a protocol which ensured that only cDNA of the desired size was selected. 30µl SPRI beads were added to the samples and incubated at room temperature for 5 minutes. The tubes were then placed on the 10x™ Magnetic Separator until the solutions became clear. At this point, cDNA of interest was in the supernatant fraction. 75µl supernatant was transferred to new tubes. 10µl SPRI beads were then added to the samples and incubated for a further 5 minutes, after which they were placed back on the 10x™ Magnetic Separator until the solutions were clear. 80µl supernatant was discarded and the beads washed with 125µl 80% ethanol in nuclease free water for 30 seconds, twice. 50.5µl EB buffer was then added to the tubes for 2 minutes, placed on the

10x™ Magnetic Separator until the solutions were clear and 50µl of the supernatant containing the cDNA placed into a new tube.

2.9.2.4 Adaptor ligation and post ligation cleanup

C1000 touch™ thermal cycler (Bio-Rad)

Ligation buffer (10x genomics)

DNA ligase (10x genomics)

Adaptor mix (10x genomics)

SPRIselect Reagent Kit (Beckman Coulter, California, USA)

10x™ Magnetic Separator (10x genomics)

80% ethanol (Merck)

Nuclease free water (Thermo Fisher Scientific)

EB Buffer (Qiagen, Hilden, Germany)

Adaptor ligation is the process of adding known synthetic oligonucleotide sequences to the end of a cDNA molecule. In this protocol adaptor ligation adds primers required for Illumina®bridge amplification sequencing. Adaptor ligation was achieved by adding 17.5µl nuclease free water, 20µl ligation buffer, 10µl DNA ligase and 2.5µl adaptor mix to 50µl of purified cDNA sample. This was incubated in a thermal cycler at 30°C for 15 minutes and 20°C for a further 15 minutes. To get rid of any excess reagents the samples were then cleaned using SPRI beads. 80µl was added to the samples and incubated for 5 minutes at room temperature. After incubation on the 10x™ Magnetic Separator until solutions became clear, the supernatant was then removed and the samples washed in 200µl 80% ethanol in nuclease free water 2 times for 30 seconds. The samples were allowed to air dry for 2 minutes and 30.5µl EB buffer added. This was left for a further 2 minutes and the samples placed onto the 10x™ Magnetic Separator until the solutions were clear. 30µl purified cDNA was then transferred to a new tube.

2.9.2.5 Sample index PCR

C1000 touch™ thermal cycler (Bio-Rad, California, USA)

PN220103 Chromium™ i7 sample index plate (10x genomics)

Nuclease free water (Thermo Fisher Scientific)

Amplification master mix (10x genomics)

SI-PCR primer (10x genomics)

The sample index is an oligonucleotide which is added to each sample in order to be able to identify each individual sample after the sequencing. The sample index is provided on a PN220103 Chromium™ i7 sample index plate, where each well contains a known oligonucleotide sequence, one of which is added to each sample. To achieve this, 8µl nuclease free water, 50µl amplification master mix, 2µl SI-PCR primer and 30µl purified cDNA sample was added to a PCR tube. 10µl of a Chromium i7 sample index was then added to each sample. The sample was then placed into a thermal cycler and the following protocol was run: 98°C for 45 seconds, 98°C for 20 seconds, 54°C for 30 seconds, 72°C for 20 seconds, repeated ~15 times (dependent on cDNA input), then 72°C for 1 minute and held at 4°C thereafter.

2.9.2.6 Post sample index PCR double sided size selection

SPRIselect Reagent Kit (Beckman Coulter, California, USA)

10x™ Magnetic Separator (10x genomics)

80% ethanol (Merck)

Nuclease free water (Thermo Fisher Scientific)

EB Buffer (Qiagen, Hilden, Germany)

cDNA of desired sizes was selected for in the same manner as section 2.9.2.3. 60µl SPRI beads were added to the samples and incubated for 5 minutes. The samples were then placed on the 10x™ Magnetic Separator until the solutions were clear.

150µl supernatant, containing the purified cDNA, was transferred to a new tube. 20µl SPRI beads were added to the samples and incubated for 5 minutes. The samples were again placed on the 10x™ Magnetic Separator until the solutions were clear. 165µl supernatant was removed and discarded. The samples were then washed with 200µl 80% ethanol in nuclease free water for 30 seconds. This was repeated twice. 35.5µl EB buffer was added to the samples and incubated for 2 minutes. After being placed on the 10x™ Magnetic Separator until the solution was clear, 35µl of the supernatant, containing the purified cDNA, was transferred to a new tube. The samples were then stored at -20°C until being sent for sequencing.

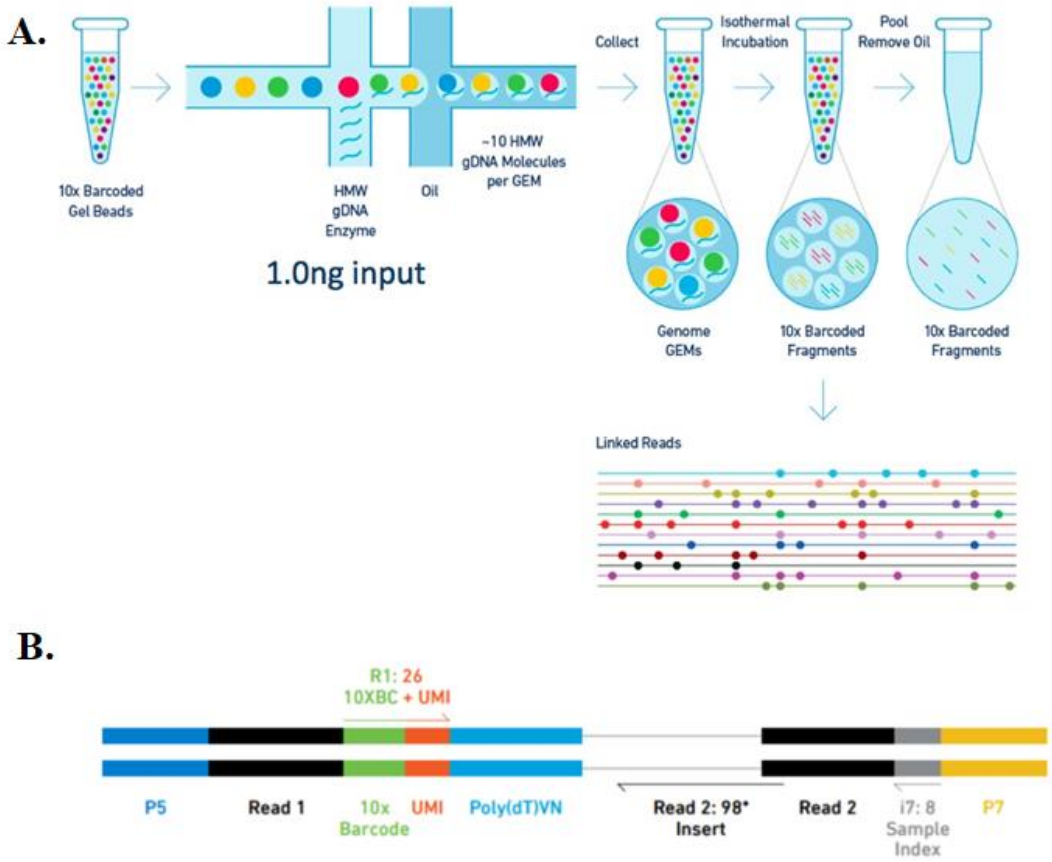


Figure 2.4. Barcoding and library preparation of cDNA samples. A: *Schematic of the cell barcoding process. The transcriptome of each cell is tagged with an oligonucleotide barcode, which is specific to each cell and can be identified during data analysis. Each cell is isolated in a nanoliter-scale Gel Bead-In Emulsion (GEM) with a 10x oligonucleotide barcode. In the GEM the cell is lysed and its RNA contents ligated with the 10x barcode. After this process, the GEMs are lysed and the oil removed from the now pooled tagged RNA. At the end of the barcoding process a pool of tagged RNA molecules from all of the cells is produced.* ***B:*** *The final sequence of a 10X library, ready for Illumina® sequencing by synthesis. The UMI (unique molecular identifier) is a 10 nucleotide sequence required to identify each*

experiment. The read 1, read 2 and poly-dT primer sequence are sequences added to facilitate Illumina®sequencing, as well as P5 and P7 which are required for bridge amplification during sequencing. The 10x barcode is the barcode added during barcoding of the samples which identifies each cell. The i8: 8 sample index is required to identify each sample during analysis. At the end of the library preparation process each cDNA molecule should contain all of these sequences, thus readying the cDNA for Illumina®sequencing. Adapted from www.10xgenomics.com.

2.9.2.7 Quality control of libraries

Quality control (QC) was performed on the libraries by Dr Pam Brown in the Queens Medical Research Institute Biomolecular Core using a LabChip® GX Touch 24 Nucleic Acid Analyzer (Perkin Elmer, Buckinghamshire, UK). QC data was assessed by Dr Beth Henderson (CIR) and all samples deemed of sufficient quality to take forward for sequencing. QC analysis was also carried out by Edinburgh Genomics before sequencing so as to ensure the samples were of sufficient quantity, this analysis is summarised in *table 2.4*.

Sample	Concentration (ng/µL)	Volume (µL)	Average size (region 3) (bp)	QC Review Summary
Endometrial tissue	37.4	30	424	Passes quantity recommendations
Sham peritoneal lavage fluid	41	31	467	Passes quantity recommendations
Endometriosis peritoneal lavage fluid	76.6	32	442	Passes quantity recommendations
Endometriosis lesions	40.6	32	432	Passes quantity recommendations

Table 2.4 Summary of QC analysis on single cell RNA sequencing samples. *QC analysis was provided by Edinburgh Genomics.*

2.9.3 Illumina® sequencing of libraries

Libraries were sent to Edinburgh Genomics and Illumina® sequencing by synthesis performed using a NovaSeq 6000 sequencing system (Illumina®, San Diego, USA).

Illumina® sequencing relies on bridge amplification to amplify target cDNA *in situ*. This is achieved using a glass flowcell coated in two different types of oligonucleotides, which are complementary to P5 and P7 adaptors on the ends of the DNA added during library preparation. The adaptors on the DNA molecules bind to

the complementary oligonucleotide on the flowcell. Polymerases then amplifiy the DNA causing it to clonally expand, which form 'bridges' on the flowcell due to the fact that both ends of the DNA contain adaptors complementary to the two different types oligonucleotides (*figure 2.5*).Once amplification is complete, each individual DNA has generated a "cluster" of around 1000 copies on the flowcell. The DNA is then sequenced by incorporation of fluorescently-tagged nucleotides. Every time a nucleotide is added to the DNA molecule, the sample is excited by a laser and fluorescence is released and detected. Each base has a distinct fluorometric signature, thus the output of the sequencing is fluorescence. Base calling to transform the fluorescence output into a sequence of bases is performed which can then be taken forward to bioinformatics analysis.

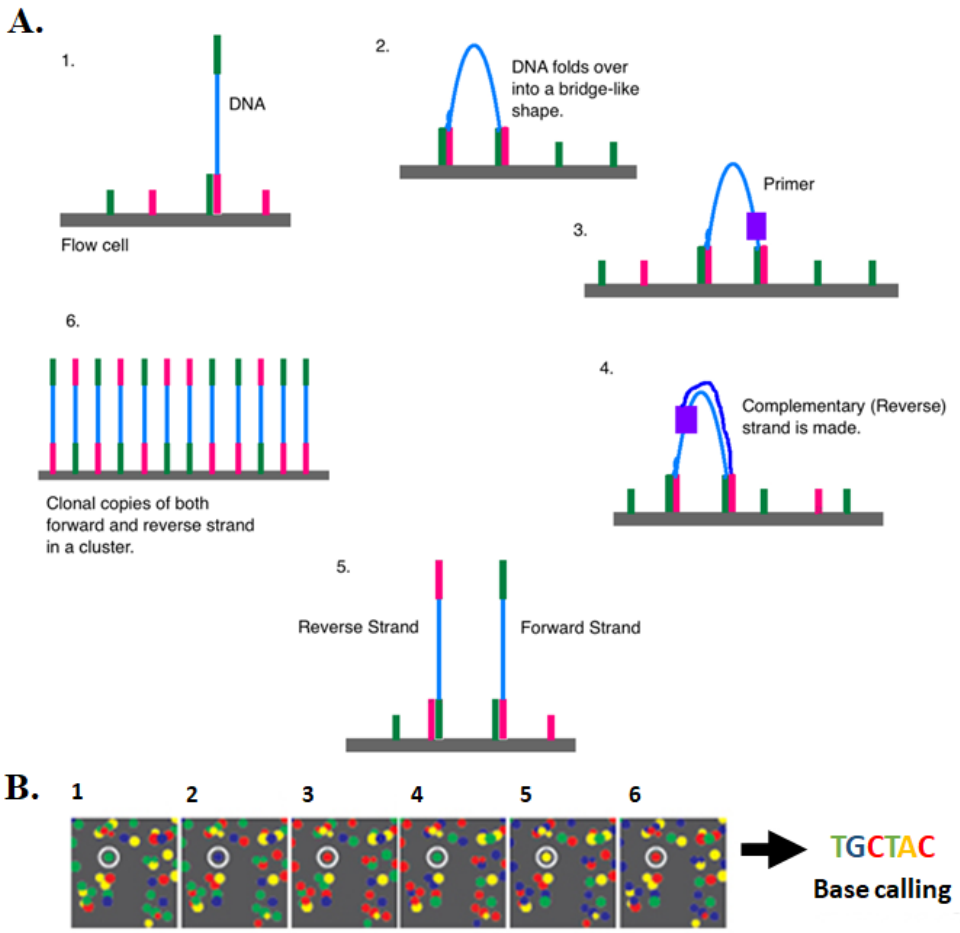


Figure 2.5. Illumina® bridge amplification sequencing. A. *Schematic diagram of Illumina® bridge amplification of DNA. The DNA forms bridges on the flowcell and is clonally amplified to create clusters of each DNA fragment.* ***B.*** *Diagram demonstrating the output of Illumina® sequencing by synthesis. Each dot on the diagram represents a cluster of clonally amplified DNA. As each nucleotide has a*

fluorescently distinct signature, the sequence of the DNA can thus be determined in the base calling process. Adapted from www.illumina.com.

2.9.4 Bioinformatics

Bioinformatics analysis was performed by Edinburgh Genomics. Bioinformatics analysis ensured all of the reads were of sufficient quality and aligned them to a reference genome (C57 BL/6 mouse). In brief, analysis was carried out using 10X Genomics' 'Cellranger' tool (version 2.0.1). FASTQ files were generated using 'Cellrangermkfastq'. 'Cellranger count' was executed for each sample, specifying the output location of 'Cellrangermkfastq', the supplied 'SI-GA' indices, and the transcriptome 'refdata-cellranger-mm10-1.2.0' as supplied by 10x. An aggregated final dataset was created using the 'Cellrangeraggr' command. The analysed files generated by Edinburgh Genomics were visualized in Loupe software and t-distributed stochastic neighbor embedding (t-SNE) plots generated. Marker genes of specific immune cells were used to cluster immune cell populations in the samples accordingly. Loupe software was then used to interrogate gene expression in the $Adgre1^{+}Itgam^{+}$ $Csf1r^{+}$ macrophage population.

Chapter 3 - Lesion resident macrophages are derived from endometrial, peritoneal and monocyte-derived macrophages

3.1 Introduction

Endometriosis is known to induce an inflammatory environment in the peritoneal cavity, for example women with endometriosis have increased concentrations of peritoneal pro-inflammatory cytokines (Giudice, 2010). Inflammation in the peritoneal cavity of mice is known to induce perturbations in peritoneal macrophage populations known as the 'macrophage disappearance reaction'. In this phenomenon, homeostatic large peritoneal macrophage numbers rapidly decrease and pro-inflammatory small peritoneal macrophage numbers increase in the peritoneal cavity (Ghosn *et al.*, 2010). Yuan *et al* reported that in mice with induced endometriosis, they observed a decrease in large peritoneal macrophages and an increase in small peritoneal macrophages in the peritoneal cavity, consistent with the fact that endometriosis lesions are thought to stimulate inflammation in the peritoneal cavity (Dunselman *et al.*, 2014). In the Edinburgh mouse model of endometriosis, we know that expression of CCL2 and CCL5 are increased in peritoneal tissue compared to control mice, suggesting that an inflammatory peritoneal environment exists in mice with induced disease (Hansen, Chalpe and Eyster, 2010). Whether perturbations in monocyte and macrophage populations in the peritoneal cavity of mice with induced endometriosis in the Edinburgh mouse model exist however is currently unknown.

We know from studies in cancer that tumour macrophages originate from both recruited monocyte-derived populations and tissue-resident cells which are seeded during foetal life (Walch *et al.*, 2014). In endometriosis lesions, we know that tissue-resident endometrial macrophages persist in endometrial tissue and contribute to the lesion macrophage population and that recruited macrophages are also present (Simón *et al.*, 1994; Garrido *et al.*, 2002; Xu *et al.*, 2015). Lesions are bathed in peritoneal fluid containing macrophages which could potentially be recruited to lesions. We also know that lesions are highly vascularised (Saraswat *et al.*, 2017) meaning monocytes could extravasate from blood vessels into lesions where they could differentiate into macrophages. The origins of the recruited macrophage population in lesions however is unknown.

3.2 Aims and hypotheses

I hypothesised that lesion macrophages are a heterogeneous population consisting of macrophages derived from eutopic endometrium as well as peritoneal and monocyte-derived macrophages.

The aims of this chapter were to:

1. Characterise peritoneal monocyte/macrophage populations in mice with induced endometriosis
2. Determine whether peritoneal and monocyte-derived macrophages are recruited to endometriosis lesions

3.3 Results

3.3.4 Endometriosis lesions induced in mice exhibit heterogeneity

Endometriosis lesions formed in the Edinburgh mouse model of endometriosis have been previously demonstrated to be macroscopically and microscopically heterogeneous, exhibiting histological similarity to lesions found in women (Simoens *et al.*, 2012; Nnoaham *et al.*, 2013). We also recently published that mice with endometriosis exhibit local and peripheral hyperalgesia, a key symptom associated with disease in humans (Bazot and Daraï, 2017; Bedaiwy *et al.*, 2017; Agarwal *et al.*, 2019). In the experiments which contributed to this thesis, mouse endometriosis lesions were most commonly found on the peritoneal wall or attached to visceral fat. Peritoneal lesions were brown/black in colour and raised from the peritoneum, commonly invading all the way through the peritoneal wall (*figure 3.1A(i)*). Lesions found attached to visceral fat were brown/yellow in colour (*figure 3.1A(ii)*). Some peritoneal lesions were filled with fluid, which was usually a reddish colour indicating the presence of blood (*figure 3.1A(iii)*).

We performed experiments using CAG-luciferase mice to identify when endometrial tissue became attached and vascularised in the peritoneal cavity. WT mice received donor endometrial tissue from CAG-luciferase mice with exhibit bioluminescent in the presence of luciferin. 24 hours after injection of endometrial tissue, WT recipient mice were injected subcutaneously with luciferin and imaged to identify

bioluminescent endometrial tissue. By injecting luciferin via a subcutaneous route, we assumed that luciferin could only reach the peritoneal cavity via the vascular system, thus endometrial tissue exhibiting bioluminescence must be vascularised. We identified concentric bioluminescent signals from all 5 mice identifying that by 24 hours lesions had become attached and vascularised in the peritoneal cavity (*figure 3.1C*).

The median number of lesions recovered from WT mice was 1 at 1 week post injection of tissue, 1 at 2 weeks post injection of tissue and 2 weeks post injection of tissue and 2 at 3 weeks post injection of tissue.

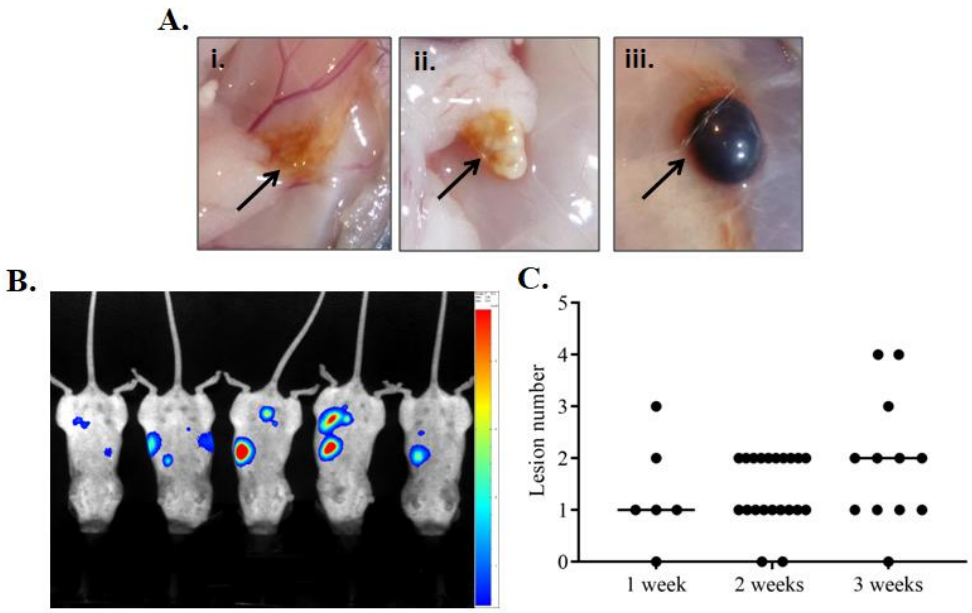


Figure 3.1. Endometriosis lesions induced in mice were attached to the peritoneal wall and fat and became established by 24 hours after injection of endometrial tissue. A: *Images of common mouse endometriosis lesions. Lesions were found on the peritoneal wall and attached to fat.* ***(i)*** *typical peritoneal lesion which had adhered to visceral fat* ***(ii)*** *lesion attached to visceral fat* ***(iii)*** *black fluid-filled lesion on the peritoneal wall* ***B:*** *Bioluminescent imaging of mice which had received CAG-Luc endometrial tissue injected into the peritoneal cavity 24 hours prior to imaging. Lesions exhibited bioluminescence upon subcutaneous injection with luciferin (n=5 mice).* ***C:*** *The number of lesions recovered from mice at 1 (n=6 mice), 2 (n=21 mice) and 3 weeks (n=12 mice) after injection of endometrial tissue. Data are expressed as median values and was analysed by analysis of variance (ANOVA).*

Mouse endometriosis lesions were subject to haematoxylin and eosin (H+E) stain and analysed for the presence of glands and stromal cells using cytokeratin and

vimentin staining respectively. H+E stain revealed that mouse endometriosis lesions had significant heterogeneity in tissue architecture. Consistent features of lesions included dense purple staining indicative of stromal cells and brown staining indicating the presence of hemosiderin, a product of macrophage activity (*figure 3.2, A-D*). Some endometriosis lesions recovered were cystic and upon cytokeratin staining contained glands with a stained epithelial layer (21% of lesions), however not all lesions had positive staining for cytokeratin (*figure 3.2, E, G, I*). All endometriosis lesions stained positive for vimentin which identified stromal cells within the tissue (*figure 3.2, F, H, J*).

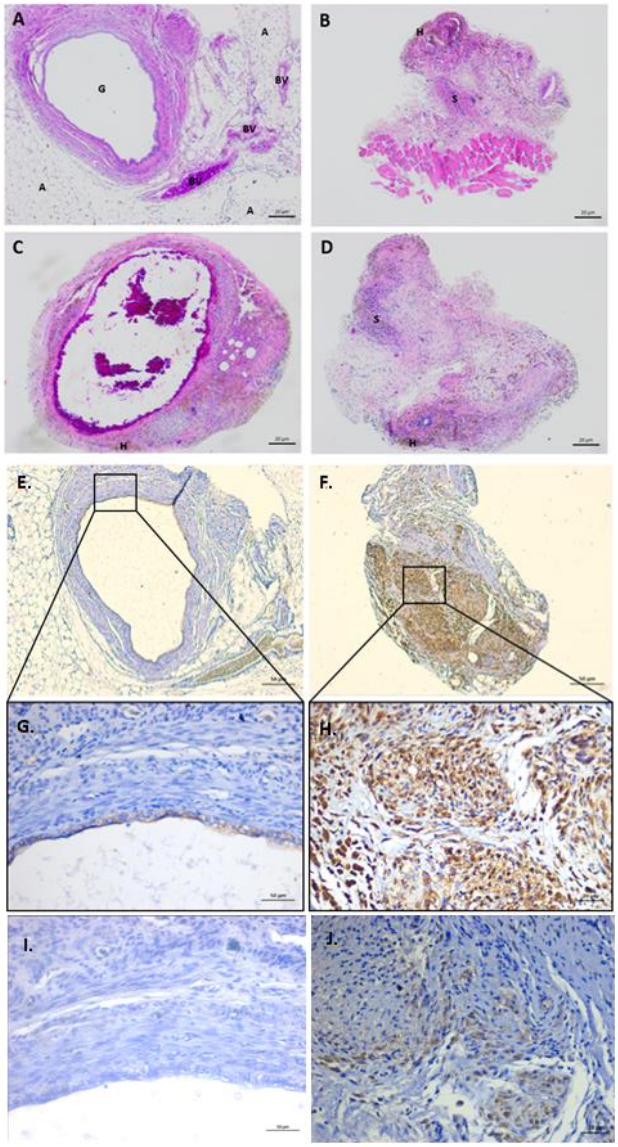


Figure 3.2. Mouse endometriosis lesions exhibited microscopic heterogeneity and all lesions stained positive for stromal cells but only a proportion of lesions had glands . *H+E stain of endometriosis lesions recovered from mice (**A-D**). Mouse lesions exhibit heterogeneity at a macroscopic and microscopic level, in line with what is observed in lesions recovered from women with endometriosis. Many lesions had brown staining, evidence of hemosiderin indicative of macrophage activity. Lesions were recovered from (**A**) fat associated with the uterus and (**B-D**) the peritoneum. A, adipose; G, gland; S, stroma; H, hemosiderin; BV, blood vessel. Cytokeratin staining on mouse endometriosis lesions identified epithelial cells and glandular compartments (**E** and **G**). Control where primary antibody was omitted showed no staining (**I**). Vimentin staining on mouse lesions demonstrated the presence of stromal cells (**F** and **H**). Control with no antibody showed no stromal cell staining. The minimal brown staining observed is likely hemosiderin (**J**).*

3.3.5 Mice with endometriosis have increased peritoneal Ly6C^{hi} monocytes

I characterised peritoneal monocyte and macrophage populations in mice with induced endometriosis to determine the effect of the presence of lesions on the peritoneal immune environment. Naive mice, sham mice and mice with endometriosis were culled at 1, 2 and 3 week time points and peritoneal lavage analysed by flow cytometry. Large and small peritoneal macrophages were delineated based on expression of MHC II and F4/80, whereby large peritoneal macrophages were MHC II^{lo} F4/80^{hi} and small peritoneal macrophages were MHC II^{lo} F4/80^{hi} (*figure 3.3A*). At a 1 week time point sham mice exhibited an increase in the large peritoneal macrophage population compared to naive but not endometriosis mice. Naive, sham and endometriosis mice all had significantly more large than small peritoneal macrophages at a 1 week time point (*figure 3.3B*). At a 2 and 3 week time point, there were no differences in large peritoneal macrophages between the groups (*figure 3.3C/D*). I did not observe any significant changes in the small peritoneal macrophage population at any time points. Next I analysed Ly6C^{hi} monocytes and Ly6C ^{hi} F4/80 ^{hi} cells transitioning from a monocyte to macrophage phenotype in the mice (*figure 3.3E*). There were no significant changes in the Ly6C^{hi} F4/80^{hi} population at any time point across the groups (*figure 3.3F-H*). There was however a significant increase in the Ly6C^{hi} monocyte population in the endometriosis group compared to naive at 1 week and compared to sham at 2 weeks. At 3 weeks there was no significant increase in the Ly6C^{hi} population in the endometriosis group.

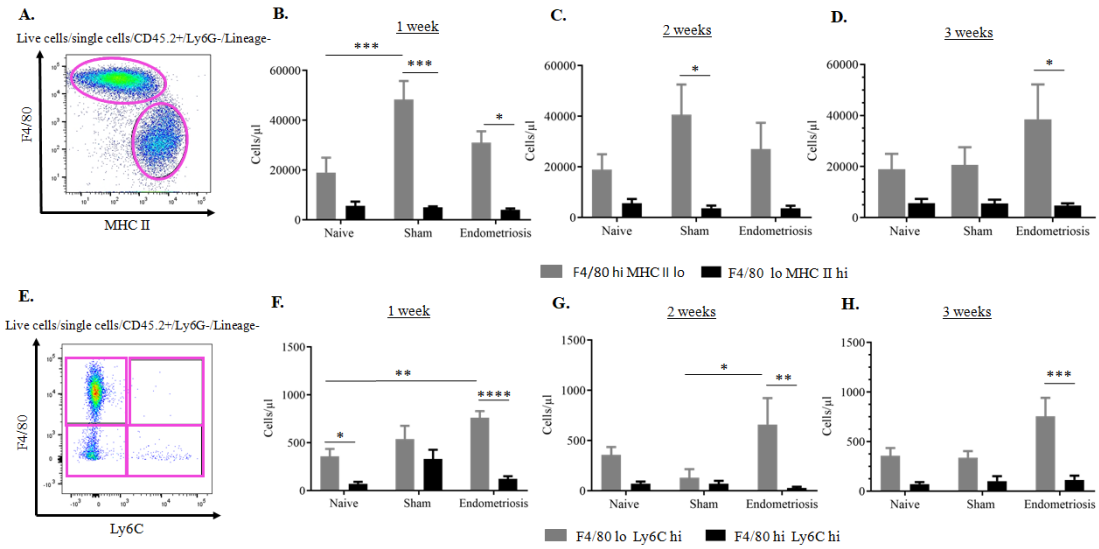


Figure 3.3. Small and large peritoneal macrophage numbers were not altered in the peritoneal lavage fluid of mice with endometriosis however an increase in Ly6C+ monocyte numbers was observed. A: *Flow plot demonstrating gating of mature peritoneal cavity macrophages (F4/80^{hi} MHC II^{lo}) and monocyte-derived small peritoneal macrophages (F4/80^{lo} MHC II^{hi}).* ***B:*** *1 week after endometrial tissue injection sham mice exhibited an increase in F4/80^{hi} MHC II^{lo} cells compared to naive which was not seen in endometriosis mice.* ***C*** *and* ***D:*** *No significant alterations in F4/80^{hi} MHC II^{lo} or F4/80^{lo} MHC II^{hi} cells were observed at 2 or 3 weeks post injection of endometrial tissue.* ***E:*** *Flow plot demonstrating gating of F4/80^{lo} Ly6C^{hi} monocytes.* ***F:*** *Mice with endometriosis exhibited an increase in F4/80^{lo} Ly6C^{hi} monocyte numbers 1 week after tissue injection compared to naive but not sham animals.* ***G:*** *At 2 weeksendometriosis mice had an increase in Ly6C^{hi} monocytes compared to sham.* ***H:*** *No difference was seen in F4/80^{lo} Ly6C^{hi} monocyte numbers at 3 weeks. 1 week time point: Naïve n=12 mice, sham n=6 mice, endometriosis n=6 mice. 2 week time point: Naïve n=12 mice, sham n=6 mice, endometriosis n=8 mice. 3 week time point: Naïve n=12 mice, sham n=8 mice, endometriosis n=16 mice. Data are expressed as means ±SEM. Analysis of variance (ANOVA), *; P < 0.05, **; P < 0.01, and ***; P < 0.001.*

3.3.6 Endometrial macrophages constitute 16% of lesion macrophages

To assess the contribution of endometrial-derived macrophages to the endometriosis lesion macrophage population, endometriosis was induced using MacGreen donor mice (macrophages are GFP+) and WT recipient mice (macrophages are GFP-) (*figure 3.4A*). GFP+ endometrial-derived macrophages were enumerated in the resultant endometriosis lesions using flow cytometry (*figure 3.4B*). Endometriosis lesions from each mouse were pooled in order to generate sufficient tissue for analysis. 16% (SEM± 4%) of lesion CD11b+ macrophages expressed GFP. The

remaining 84% (SEM±3%) were GFP- and thus were derived from the recipient mouse (*figure 3.4C*).

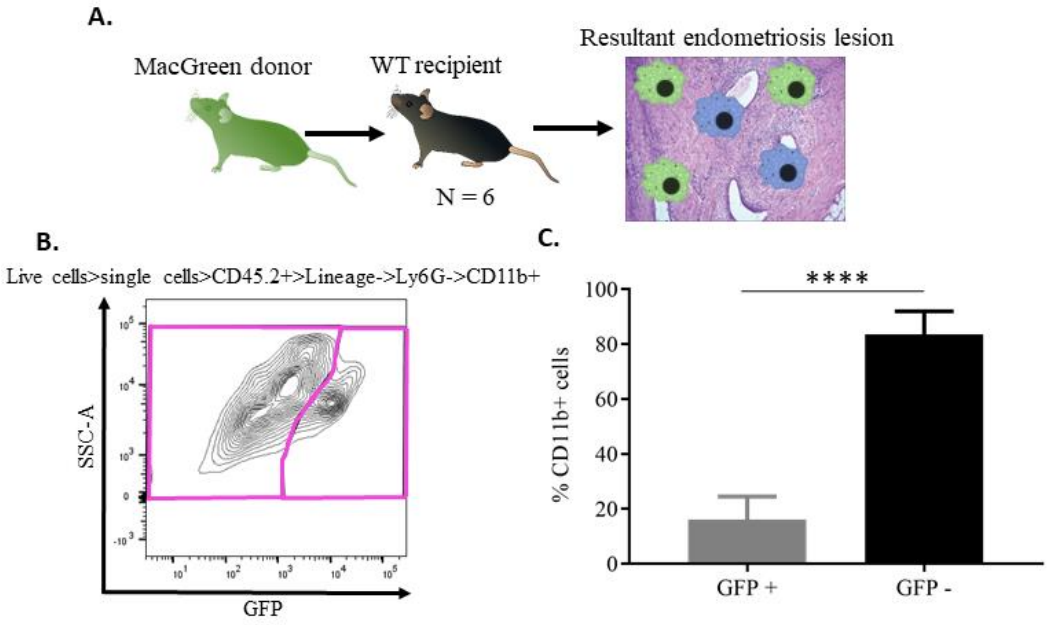


Figure 3.4. Endometrial macrophages contribute to the endometriosis lesion resident macrophage population. A: *Schematic demonstrating study design.* ***B:*** *Flow plot of GFP+ endometrial-derived CD11b+ cells in the endometriosis lesions of mice which had received donor endometrial tissue from MacGreen (Csf1r-EGFP) mice 2 weeks prior to cull (n=6 mice where lesions were pooled from each animal for analysis).* ***C:*** *GFP+ endometrial derived CD11b+ cells represented 16% (SEM± 4%) of lesion CD11b+ macrophage populations, the remaining 84% (SEM±3%) were GFP-. Significantly more CD11b+ cells were derived from infiltrating macrophage populations compared with the endometrial-derived population (p=<0.0001) (analysed by student t test). Data are expressed as means ±SEM.*

3.3.7 Large peritoneal macrophages infiltrate lesions

In order to assess the contribution of large peritoneal macrophages to endometriosis lesions, lesions were stained for the large peritoneal macrophage markers F4/80 and Gata6 (*figure 3.5*A). 48% (±SEM 4%) of cells in lesions expressed F4/80 and 4% (±SEM 2%) of these co-expressed the large peritoneal marker Gata6. A Gata6+ only population was also present in the tissue and this represented 8% (±SEM 2%) of total cells (*figure 3.5B*).

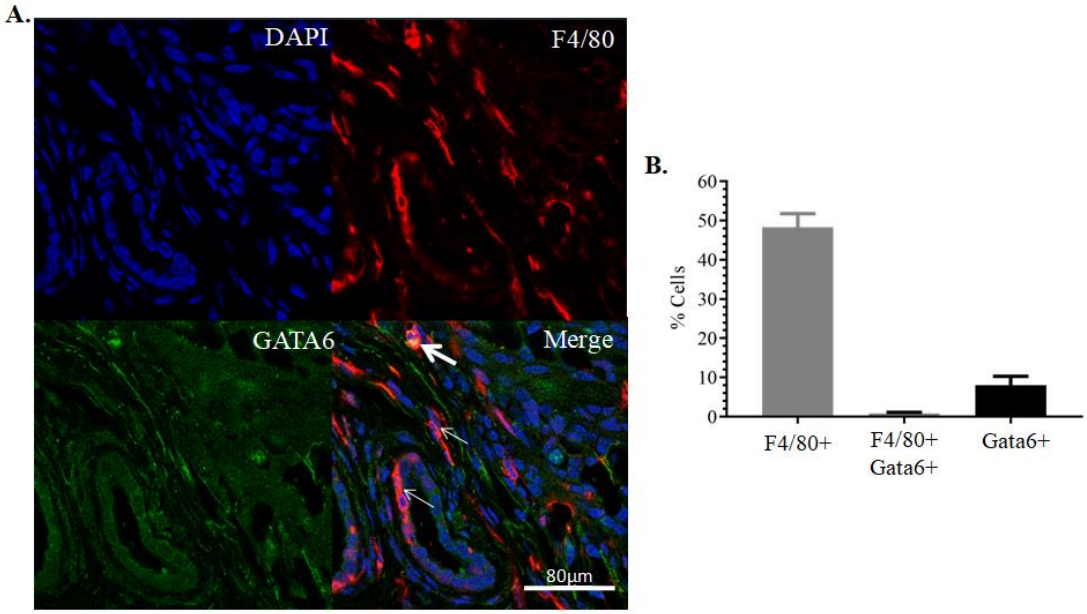


Figure 3.5. 4% of lesion resident F4/80+ cells co-expressed the large peritoneal macrophage marker Gata6. A. *Immunofluorescent stain of F4/80 and Gata6 on a mouse endometriosis lesion. Thick white arrow indicates a Gata6^{+} F4/80^{+} cell. Thin white arrows indicate single F4/80^{+} cells.* ***B.*** *48% (±SEM 4%) of cells in lesions expressed F4/80. 2% (±SEM 1%) of cells co-expressed both F4/80 and Gata6 and these constituted 4% (±SEM 2%) of the F4/80 population. Cells only expressing Gata6 could also be detected and contributed to 8% (±SEM 2%) of total cells. Values expressed as % of total DAPI^{+} cells. N=10 lesions were stained which were isolated from n=7 animals. Data are expressed as means ± SEM.*

Large and small peritoneal macrophages were isolated from the peritoneal cavity of MacGreen mice using FACS of peritoneal lavage fluid isolated from the mice at cull. Large (F4/80 ^{hi} MHC II ^{lo}) and small (F4/80 ^{lo} MHC II ^{hi}) peritoneal macrophages were gated as previously described (*figure 3.6B*). Large or small peritoneal macrophages sorted by FACS were adoptively transferred into the peritoneal cavity of endometriosis mice (*figure 3.6A*). To confirm the propagation of viable macrophages into the peritoneal cavity, two weeks after adoptive transfer mice were culled and peritoneal lavage fluid analysed by flow cytometry for detection of GFP+ adoptively transferred cells. Viable GFP+ macrophages were detected in the peritoneal cavity of all mice which had received adoptive transfer and this population was absent in sham control mice (*figure 3.6C*).

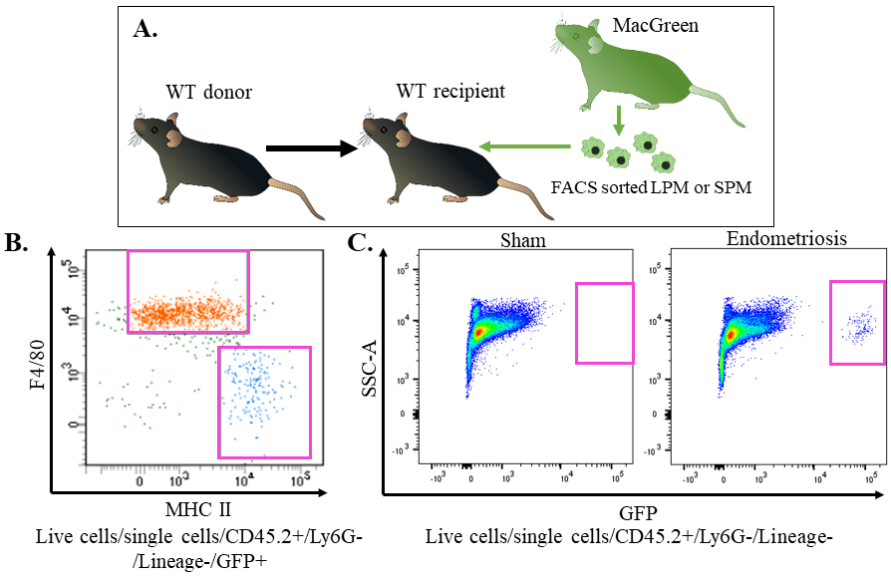


Figure 3.6. GFP+ small or large peritoneal macrophages were adoptively transferred into the peritoneal cavity of endometriosis mice which persisted and survived in the cavity for 2 weeks . A: *Schematic of study design.* ***B:*** *FACS plot of sorted GFP+ LPM (F4/80 hi MHC II lo) and small peritoneal macrophage (F4/80 lo MHC II hi) populations.* ***C:*** *Flow plots of GFP+ macrophages in mouse peritoneal lavage fluid. Live GFP+ macrophages were detected in all mice 2 weeks after adoptive transfer (n=12 mice), compared to sham animals where no GFP+ cells were detected (n=22 mice) (P=0.026, student t test).*

At the time of cull, endometriosis lesions were isolated and stained for GFP to assess infiltration of adoptively transferred GFP+ large or small peritoneal macrophages into the tissue. Lesions isolated from mice which had received GFP+ large peritoneal macrophages had an absence of GFP+ cells in peritoneal tissue surrounding the lesion, however GFP+ cells were observed within lesion tissue (*figure 3.7A (i)*). GFP+ cells within lesions aggregated around circular structures (*figure 17A (ii)*) and formed clusters within the tissue (*figure 3.7A (iii)*). GFP+ cells were also identified accumulated around areas with the histological phenotype of necrotic tissue which had a low abundance of DAPI+ cells (*figure 3.7B*).

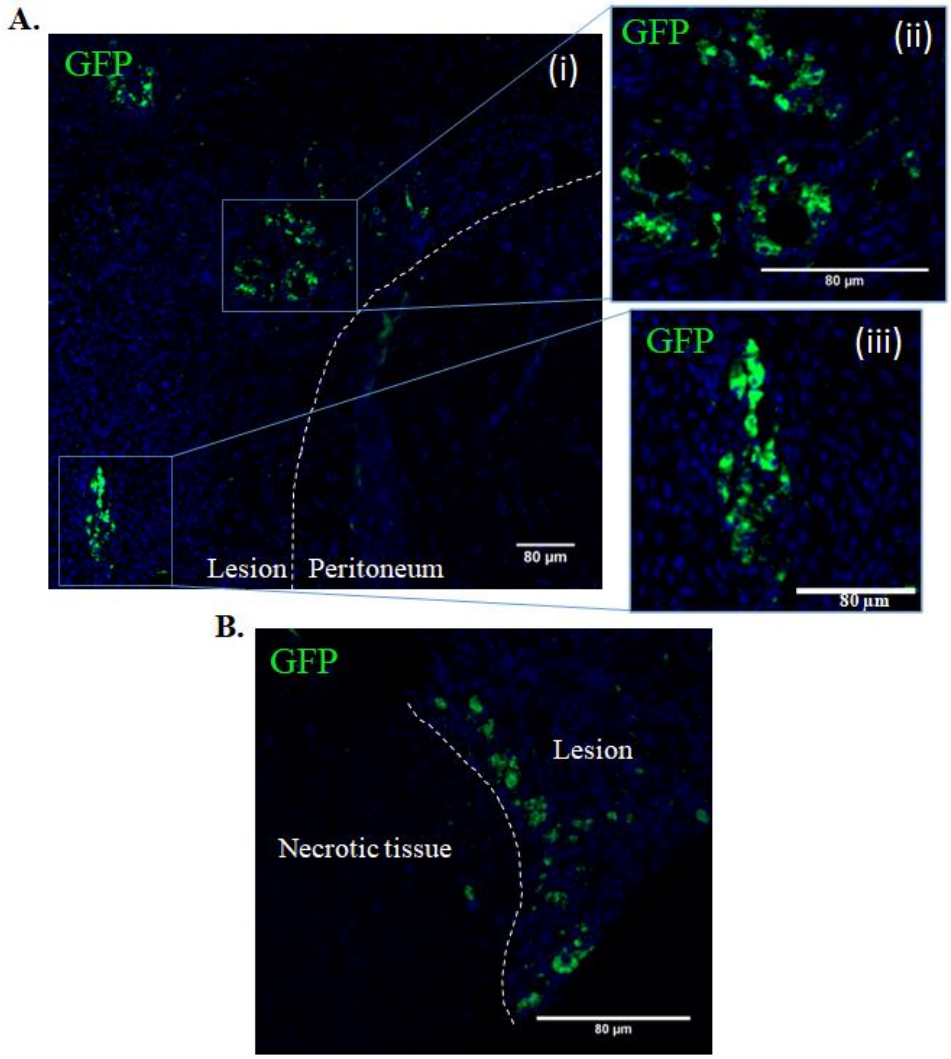


Figure 3.7. GFP+ large peritoneal macrophages infiltrate endometriosis lesions and form clusters of cells in lesion tissue.. A: (i) *GFP immunofluorescent stain on a mouse endometriosis lesion which had received adoptive transfer of GFP+ LPM into the peritoneal cavity. GFP+ cells were not observed in surrounding peritoneal tissue but were observed in lesion tissue.* ***(ii)*** *GFP+ large peritoneal macrophages formed clusters around circular structures within lesions.* ***(ii)*** *GFP+ large peritoneal macrophages formed a cluster of cells within lesion tissue.* ***B:*** *GFP+ large peritoneal macrophages accumulated at the edge endometriosis lesion tissue next to tissue which has the histological characteristics of necrotic tissue due to a low abundance of DAPI+ cells. N=12 mice.*

GFP staining on lesions from mice which had received adoptive transfer of small peritoneal macrophages revealed that GFP+ cells were not present in lesion tissue (*figure 3.8A*). GFP+ cells were only present in peritoneal tissue around the lesion (*figure 3.8B*). GFP+ cells in the peritoneal tissue had a circular morphology consistent with the fact that small peritoneal macrophages are derived from monocytes, which exhibit a similar circular morphology.

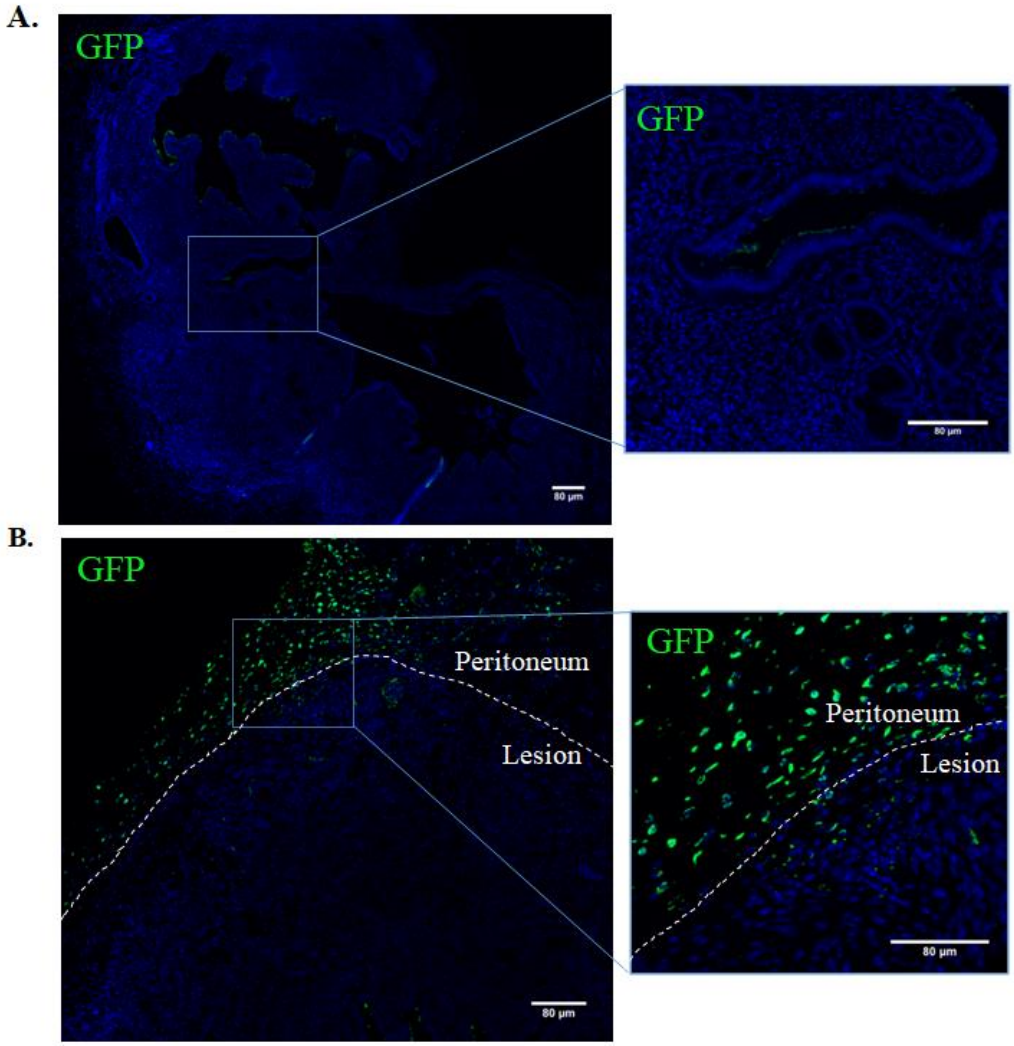


Figure 3.8. GFP+ small peritoneal macrophages did not infiltrate mouse endometriosis lesions but accumulated in surrounding peritoneal tissue. A: *Immunofluorescent GFP staining on a mouse endometriosis lesion which had received adoptive transfer of GFP+ small peritoneal macrophages. No GFP staining was observed in the lesion.* ***B:*** *GFP+ small peritoneal macrophages were observed in peritoneal tissue surrounding endometriosis lesion tissue but there were no GFP+ cells in the lesions. N = 12 mice.*

Thus, I have shown for the first time that large peritoneal macrophages but not small peritoneal macrophages infiltrate endometriosis lesions.

3.3.8 Ly6C^{hi} monocytes and Ly6C^{hi} F4/80^{hi} monocyte-derived macrophages contribute to endometriosis lesions

To ascertain whether Ly6C^{hi} monocytes and Ly6C^{hi} F4/80^{hi} monocyte-derived macrophages contribute to the lesion microenvironment in our model, dual immunofluorescence for Ly6C and F4/80 was performed on endometriosis lesions biopsies from endometriosis mice. Ly6C+ monocytes, F4/80+ Ly6C+ monocyte-derived macrophages and F4/80+ macrophages were present in endometriosis lesion tissue as well as in the peritoneum (*figure 3.9A/B*). The number of positive cells stained for the Ly6C and F4/80 markers was enumerated in the peritoneal and lesion tissue using Definiens Architect XD^{TM} software. There were a similar number of Ly6C+ monocytes, F4/80+ Ly6C+ monocyte-derived macrophages and F4/80+ macrophages in peritoneal tissue compared with endometriosis lesion tissue, however there was a modest increase in the number of F4/80+ macrophages in the lesions (*figure 3.9C*).

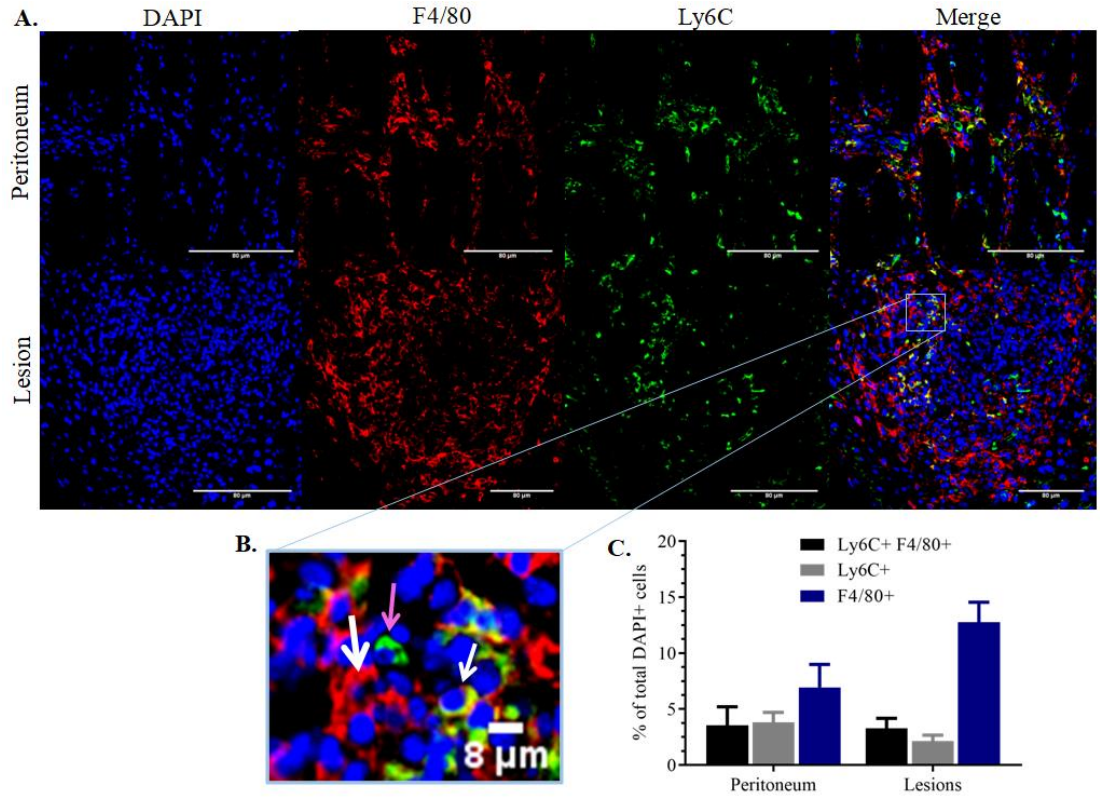


Figure 3.9. Immunofluorescent staining on mouse endometriosis lesion and peritoneal tissue demonstrated infiltration of Ly6C+ monocytes and Ly6C+ F4/80+ monocyte-derived macrophages into tissue. ***A:*** *F4/80 and Ly6C dual immunofluorescence on mouse endometriosis lesion and peritoneal tissue.* ***B:*** *Ly6C+ cells (thick pink arrows), F4/80+ Ly6C+ cells (thick white arrows) and F4/80+ cells (thin white arrows) were present in endometriosis lesion tissue.* ***C:*** *Numbers of F4/80+, Ly6C+ and F4/80+ Ly6C+ cells in peritoneal tissue adjacent to lesions (n=6 lesions from n=6 mice) and lesion tissue (n=9 lesions from n=9 mice). There were no significant changes in cell number however a modest increase in F4/80+ cells was observed in endometriosis lesions compared to adjacent peritoneal tissue. Data are expressed as means ± SEM and analysed by student t test.*

Using Definiens Architect XD™ software, the tissue was first assigned as either lesion, peritoneum, haemorrhage or adipose tissue (*figure 3.10A*). Haemorrhage and adipose tissue were removed from the analysis and cells in the lesion and peritoneal tissue analysed. Subsequent images were generated which included only lesion and peritoneal tissue and highlighted each individual cell as a circle. These circles were artificially coloured dependent on the markers the cell expressed. F4/80+ cells are shown as blue dots, Ly6C+ cells as green dots, F4/80+ Ly6C+ cells as red dots and F4/80- Ly6C- cells as grey dots (*figure 3.10B*). These images allowed the distribution of cells across the tissue to be more easily visualised and demonstrated

that F4/80+ and F4/80+ Ly6C+ cells formed clusters throughout specific areas of the tissue and were not homogeneously distributed.

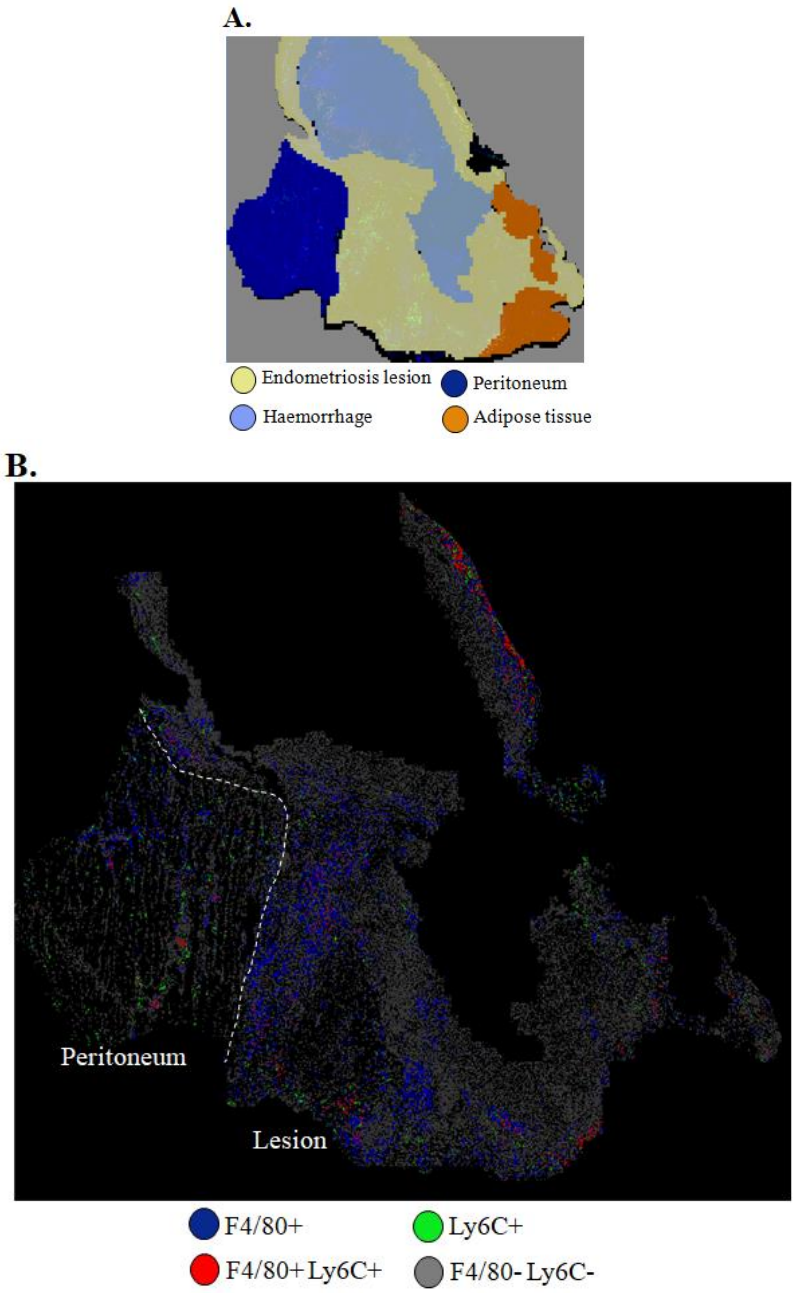


Figure 3.10. Monocyte and macrophage distribution in endometriosis lesion tissue was not homogenous . A: *Using Definiens Architect XD™ software, mouse endometriosis tissue biopsy samples stained for F4/80 and Ly6C were separated based on tissue type and defined as either endometriosis lesion (beige), peritoneum (dark blue), area of haemorrhage (light blue) or adipose tissue (orange). Cell numbers in peritoneal and lesion tissue were enumerated.* ***B:*** *Whole tissue image showing peritoneal and lesion tissue only, demonstrating the distribution of F4/80+ cells (blue dots), Ly6C+ cells (green dots), F4/80+ Ly6C+ cells (red dots) and F4/80- Ly6C- cells (grey dots) across the tissue. F4/80+ and F4/80+ Ly6C+ cells*

formed specific clusters throughout the tissue and were not homogeneously distributed within lesions.

Figure 3.11 shows different areas of the tissue section from *figure 3.10* and demonstrates the differential distribution of Ly6C+, Ly6C+ F4/80+ and F4/80+ cells across the tissue. Some sections were very densely infiltrated by F4/80+ macrophages and these areas also contained a lower number of Ly6C+ monocytes and Ly6C+ F4/80+ monocyte-derived macrophages (*figure 3.11A (i)*). Other areas of the tissue had dense clusters of F4/80+ macrophages, Ly6C+ monocytes and Ly6C+ F4/80+ monocyte-derived macrophages which interestingly clustered together at the edge of the lesion tissue (*figure 3.11A (ii)*). In the peritoneal tissue, F4/80+ macrophages, Ly6C+ monocytes and F4/80+ Ly6C+ monocyte-derived macrophages were also present throughout the tissue and smaller clusters of cells were present (*figure 3.11A (iii*)).

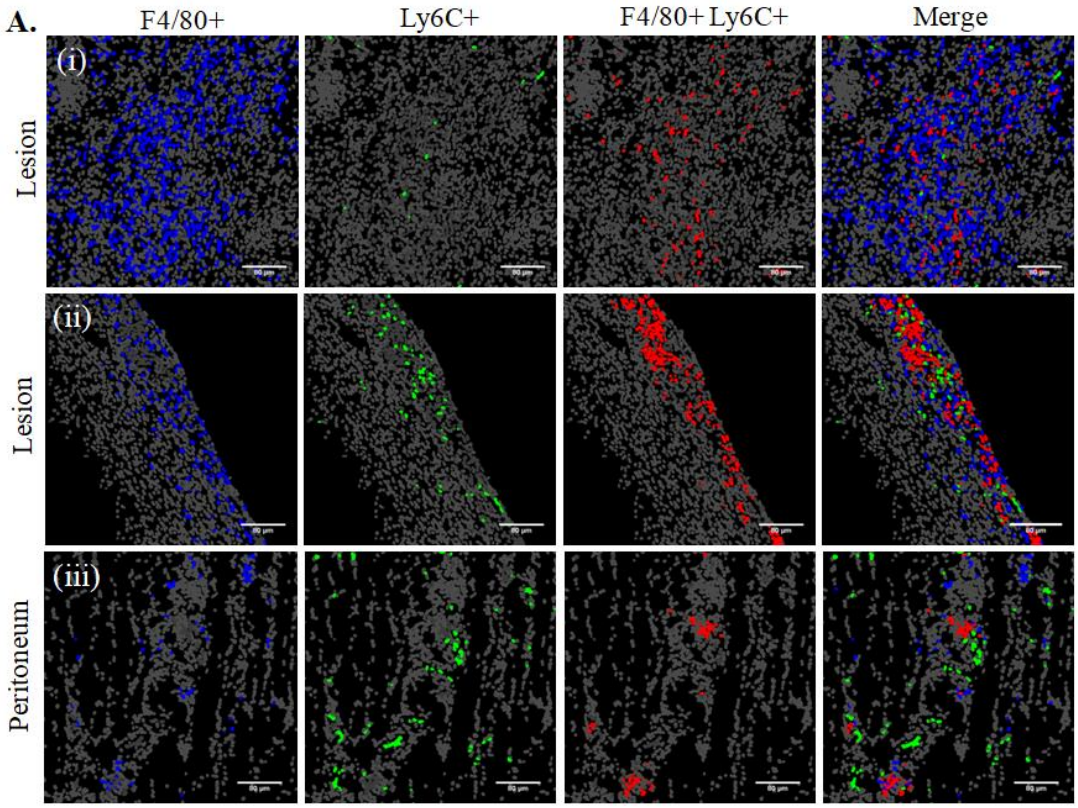


Figure 3.11. The distribution of F4/80+, Ly6C+ and F4/80+ Ly6C+ cells in endometriosis lesions was heterogeneous. *The distribution of F4/80+, Ly6C+ and F4/80+ Ly6C+ cells in endometriosis lesions was heterogeneous dependent on the area of the lesion A (i, ii). A (iii): The distribution of F4/80+, Ly6C+ and F4/80+ Ly6C+ cells in peritoneal tissue.*

Peritoneal endometriosis lesions analysed were heterogeneous in the monocyte/macrophage populations present within the tissue. *Figure 3.13* shows a peritoneal endometriosis lesion which was largely infiltrated by F4/80+ macrophages and a lower number of Ly6C+ monocytes, however Ly6C+ F4/80+ monocyte-derived macrophages were almost absent from the tissue. Lesion tissue was highly infiltrated with F4/80+ macrophages which were not found in adjacent peritoneal tissue. There appeared to be areas histologically similar to necrosis in the tissue, which are indicated with an asterix (*figure 3.12B*). F4/80+ macrophages seemed to be clustered around necrotic areas of tissue within the lesion (*figure 3.12B*).

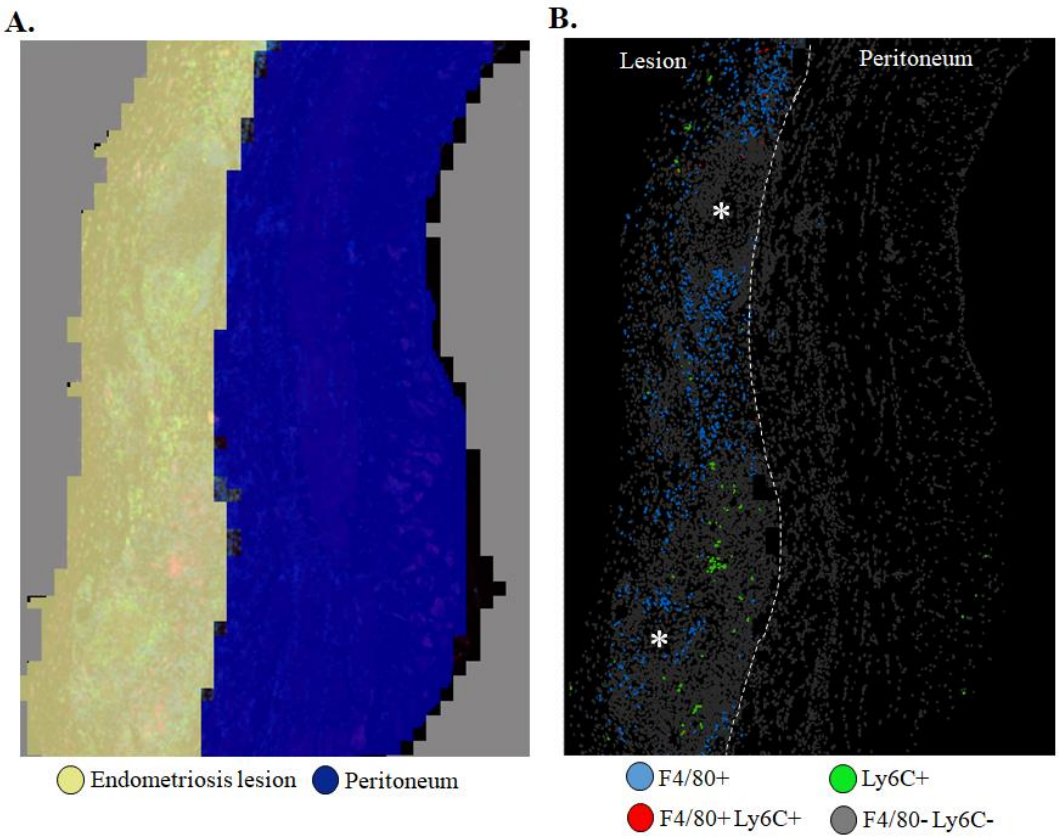


Figure 3.12. F4/80+ macrophages infiltrated lesions and accumulated around areas characteristic of necrotic tissue. A: *Characterisation of an endometriosis mouse tissue biopsy using Definiens Architect XD™ software showing areas of endometriosis lesion tissue (beige) and peritoneal tissue (dark blue).* ***B:*** *Ly6C and F4/80 expression across the tissue section. F4/80+ cells (light blue) were higher in number in lesion tissue compared to peritoneal tissue and appeared to cluster around areas with the histological characteristic of necrotic tissue (indicated with an asterix). Ly6C+ cells (green) were also present in the lesion however F4/80+ Ly6C+ cells were of low abundance.*

3.4 Summary

In this chapter, I have demonstrated that mice with induced endometriosis have similar large and small peritoneal macrophage numbers to sham controls but an increase in the number of Ly6C^{hi} monocytes in the peritoneal cavity. I have also characterised the ontogeny of lesion macrophages and revealed that this population is derived from endometrial tissue-resident macrophages, as well as recruited large peritoneal macrophages and monocyte-derived macrophages. Small peritoneal macrophages did not become incorporated into the endometriosis lesions in my model. Image analysis of Ly6C and F4/80 staining on lesion tissue revealed that monocytes and macrophages formed clusters throughout the tissue and were not homogeneously distributed.

3.5 Discussion

I my studies, mice with induced endometriosis had similar numbers of large and small peritoneal macrophages compared to sham animals. This finding contradicts a paper from Yuan *et al* which reported perturbations in peritoneal macrophage populations (Vigano *et al.*, 2018). The key difference between these studies was the mouse model used. Yuan *et al* isolated naive endometrial tissue and injected this into the peritoneal cavity of recipient mice which had intact ovaries, however in my model 'menses-like' endometrial tissue was generated and injected into the peritoneal cavity of mice which had undergone ovariectomy and been supplemented with estradiol valerate (Greaves *et al.*, 2014; Yuan *et al.*, 2017b). In the Edinburgh mouse model I identified a modest increase in the LPM population and this could be due to the superphysiological levels of estrogen utilised in the model and the pro-proliferative effect estrogen is known to have on macrophages (Greaves *et al.*, 2014; Pepe *et al.*, 2017). Hence, peritoneal macrophage populations may be perturbed by estrogen levels in our model, indeed, sham mice also demonstrated an increase in the LPM population, supporting this notion. It is also possible that due to the differential endometrial tissue utilised in these models, a divergent inflammatory response was generated in the peritoneal cavity. We know that ~90% of women experience cyclic retrograde menstruation (Sampson, 1927), meaning the peritoneal cavity may be consistently challenged with menses endometrial tissue. It is possible therefore that

peritoneal cavity immune cells are tolerogenic to menses endometrial tissue to avoid cyclic peritoneal inflammation in women. However, mice do not menstruate or experience retrograde menstruation, suggesting that this immunotolerance may be regulated by factors present in decidualised endometrial tissue rather than immune cells in the peritoneal environment.

There was, however, an increase in peritoneal Ly6C^{hi} inflammatory monocytes in the Edinburgh mouse model, suggesting that endometriosis lesions produce a chemotactic signal for these cells, causing their extravasation from blood vessels into the peritoneal cavity. In mice, the extravasation of Ly6C^{hi} monocytes is regulated by CCR2 (Lu *et al*, 2002), and we know that peritoneal biopsies from mice with endometriosis show an up-regulation of CCL2 (Greaves *et al.*, 2014), suggesting that CCL2 could be responsible for the increase in Ly6C^{hi} monocytes observed in this model. Studies in cancer suggest that pathogenic tissue can modulate Ly6C^{hi} monocyte recruitment and function to exacerbate disease. In a mouse model of breast cancer, Ly6C^{hi} inflammatory monocytes were recruited to metastatic sites where they facilitated tumour metastasis by secretion of VEGF. Recruitment to the tumour was modulated by CCL2 which was secreted by the tumour and stroma (Qian *et al.*, 2011). It is possible therefore that endometrial stromal cells may secrete CCL2 to recruit inflammatory monocytes to endometriosis lesions, which may exacerbate disease. However, the role of this population in endometriosis lesion development has not been defined.

Endometrial macrophages persist in donor endometrial tissue to constitute 16% of the lesion-resident macrophage population. In the endometrium, macrophages play roles in breakdown of the functional layer of the endometrium as well as repair and remodelling (Mahmood and Templeton, 1991; Johnson *et al.*, 2017). Endometrial macrophages are also implicated in angiogenesis in the endometrium by secretion of VEGF (Canis *et al.*, 1997). In women, ectopic endometrial tissue (lesions) are vascularised and undergo cyclic shedding and repair, akin to the eutopic endometrium (Vercellini *et al.*, 2006; Ek *et al.*, 2015). It is possible therefore that endometrial-derived macrophages in ectopic endometrial tissue play similar roles in tissue remodelling and angiogenesis as they do within the eutopic endometrium.

Conversely, endometrial-derived macrophages in endometriosis lesions are exposed to a differential microenvironment compared with the eutopic endometrium. This novel microenvironment has the potential to modulate endometrial-derived macrophage phenotype and function, however the phenotype and function of endometrial macrophages in endometriosis lesions is currently unknown.

In this chapter, I demonstrated that large, but not small, peritoneal macrophages become incorporated into endometriosis lesions. Under inflammatory stimuli, small peritoneal macrophages are rapidly recruited into the peritoneal cavity where they produce a number of pro-inflammatory cytokines to mount an inflammatory response (Cassado, D'Império Lima and Bortoluci, 2015; Rückerl *et al.*, 2017). The fact that women and mice with endometriosis have homeostatic numbers of small peritoneal macrophages suggests that a traditional inflammatory response is not mounted by the presence of endometriosis lesions. In this way, ectopic endometrial tissue is able to escape immune clearance. This is analogous to the absence of small peritoneal macrophages in lesion tissue, and suggests that the inability of small peritoneal macrophages to respond to and invade ectopic endometrial tissue may contribute to pathology.

Conversely, large peritoneal macrophages become incorporated into lesions and are found throughout the lesions. In a sterile liver injury model in mice, large peritoneal macrophages rapidly infiltrated the liver where they were involved in tissue remodelling and regeneration (Wang and Kubes, 2016). It is possible therefore that large peritoneal macrophages play roles in wound healing and tissue remodelling in lesions, potentially maintaining pathogenic endometrial tissue as opposed to 'healthy' peritoneal tissue. Large peritoneal macrophages in lesions form clusters which are frequently around circular structures. These circular regions could represent remodelling glands, although this has not been identified. The specific localisation of large peritoneal macrophages suggests that they may be fulfilling a function in this area of the lesion, however this is currently unknown.

In addition, I demonstrated that monocytes and monocyte-derived macrophages contribute to the endometriosis lesion microenvironment. Ly6C+ inflammatory monocytes and Ly6C+ F4/80+ cells which are transitioning from a monocyte to

macrophage phenotype were found in lesion tissue at a similar frequency to the peritoneum. However, these results were confounded by the fact that these cells formed clusters within the lesion tissue, meaning that in certain regions of the lesion monocyte and macrophage numbers were significantly higher than the peritoneum, however this was attenuated by areas with little monocyte and macrophage infiltration. In tumours, microenvironments exist which are infiltrated by macrophages which play distinct roles in disease (Quatromoni and Eruslanov, 2012). Here I have shown multiple origins and diverse distributions of myeloid populations within endometriosis lesion tissue in our model, suggesting existence of difference phenotypes in discrete locations within lesions.

Chapter 4 - Ly6C+ monocytes can be recruited to endometriosis lesions independent of CCL2

4.1 Introduction

In chapter 3, I demonstrated that macrophages within endometriosis lesions are derived from endometrial macrophages, large peritoneal macrophages and monocyte-derived macrophages. The roles that these differential populations play in disease are currently unknown. The use of clodronate liposomes to deplete peritoneal phagocytic macrophages has been demonstrated to reduce the number of lesions which form in mice (Forster*et al.*, 2019). Large peritoneal macrophages are highly phagocytic compared to small peritoneal macrophages (Ghosn *et al.*, 2010), suggesting a bias in depletion of this population. This depletion method also encompasses phagocytic monocyte-derived macrophages however and thus cannot differentiate between these populations. In cancer, macrophages with differential ontogenies play distinct roles in tumour development (Noy and Pollard, 2014; Zhu *et al.*, 2017), suggesting that macrophages with different origins in endometriosis could also play diverse roles in disease.

In the previous chapter I also demonstrated that mice with endometriosis exhibit increased peritoneal Ly6C^{hi} monocytes. Monocytes are normally recruited into tissues by CCL2 (Wynn, Chawla and Pollard, 2013), a chemokine which has also been shown to be up-regulated in the peritoneum and lesions of mice with endometriosis (Greaves *et al.*, 2014). Despite numerous descriptive studies identifying chemokine's associated with endometriosis (Pizzo *et al.*, 2002), the pathways that regulate monocyte and macrophage recruitment have not been mechanistically interrogated.. To explore the CCR2-CCL2 chemokine pathway in the recruitment of inflammatory monocytes/monocyte-derived macrophages to endometriosis lesions we used. CCR2 and CCL2 null mice.CCR2 null mice have a very low abundance of circulating Ly6C^{hi} monocytes due to inhibited mobilisation from the bone marrow, a process regulated by CCR2 (Boring *et al.*, 1997). These mice also have a very low number of peritoneal Ly6C^{hi} monocytes, Ly6C^{hi} monocyte-derived macrophages and small peritoneal macrophages, which are derived from Ly6C^{hi} monocytes, due to their inability to extravasate into the cavity (Boring *et al.*, 1997). CCL2 null have normal peritoneal macrophage numbers but an

inability to recruit Ly6C^{hi} monocytes under inflammatory conditions (Lu *et al.*, 2002).

4.2 Aims and hypotheses

I hypothesised that macrophage populations with different ontogenies play differential roles in lesion development, and that CCL2 is an important chemokine for regulating recruitment of Ly6C^{hi} monocytes and monocyte-derived macrophages in endometriosis.

The aims of this chapter were to:

1. Investigate the role of endometrial and peritoneal macrophages in the development of endometriosis lesions
2. Determine the importance of the CCL2-CCR2 signalling pathway in the recruitment of Ly6C^{hi} monocytes and monocyte-derived macrophages to endometriosis lesions

4.3 Results

4.3.1 Endometrial macrophage depletion did not affect lesion number

To investigate the role of endometrial macrophages in endometriosis lesion development, CSF1R cKO mice were used as donors in our mouse model to generate macrophage depleted decidualised endometrial tissue (*figure 4.1A*). Upon treatment with doxycycline (2μg/ml in 5% sucrose water), Cre recombinase selectively knocked out the Csf1r gene, causing depletion of Csf1r expressing macrophage populations (Li *et al.*, 2006). Doxycycline was administered from days 15-19 in the Edinburgh mouse model protocol (refer to *figure 1.3* for protocol timeline). These days of the protocol correlate with the generation of decidualised endometrial tissue through intrauterine injection of sesame oil and P4 pellet withdrawl. Compared to wild type mice, CSF1R cKO mice had a 34% (± SEM 13%) depletion in F4/80^{hi} Ly6C^{lo} mature macrophages and a 32% (± SEM 18%) depletion in F4/80^{lo} Ly6C^{hi} monocytes (*figure 4.1B*) in decidualized endometrial tissue. No significant changes in Ly6G+ neutrophils were observed (*figure 4.1C*). Endometriosis was induced in WT mice with either macrophage depleted (CSF1R cKO) or non-depleted (WT) tissue. There was no

significant difference in the number of lesions recovered from each group (*figure 4.1D*). However, 4 out of 9 mice in the macrophage depleted group developed no endometriosis lesions but all mice in the group which received non-depleted tissue developed lesions (n=10) (*figure 4.1D*). Lesions recovered from mice which had received macrophage-depleted tissue were smaller, although not significantly, from the group which had received non-depleted tissue (*figure 4.1E*).

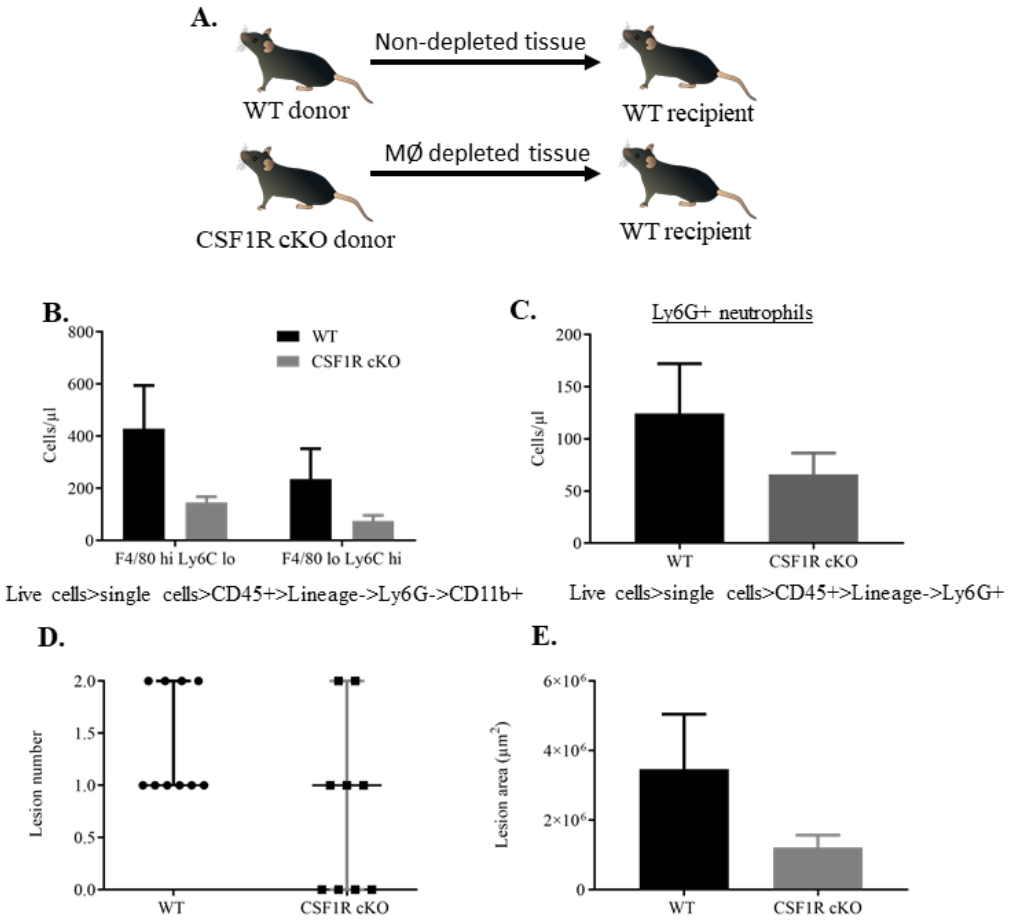


Figure 4.1. Endometrial macrophage depletion in donor endometrial tissue did not significantly alter the number or size of lesions formed in mice . A: *Schematic demonstrating study design.* ***B:*** *Flow cytometry data demonstrating depletion of macrophage populations in WT (n=4) and Csf1r cKO (n=5) donor endometrial tissue. Treatment of donor Csf1r cKO mice with doxycycline caused a 34% (± SEM 13%) depletion in the F4/80^{hi} Ly6C^{lo} mature macrophage population and a 32% (± SEM 18%) depletion in the F4/80^{lo} Ly6C^{hi} monocyte population in the decidualised endometrium.* ***C:*** *There were no significant differences in Ly6G^{+} neutrophils in WT (n=4) or Csf1r cKO (n=5) donor endometrial tissue.* ***D:*** *Lesion numbers from mice which had received either WT (n=10) or macrophage depleted, Csf1r cKO tissue (n=9). Data are shown as median + 95% confidence intervals.* ***E:*** *No significant difference in lesion size was observed between groups, however a modest reduction*

in lesion size was observed in mice which had received Csf1r cKO macrophage depleted tissue. Data are expressed as means ± SEM for all graphs except for ***D*** *where median is shown. Analysed by Mann-Whitney U test.*

4.3.2 Large or small peritoneal macrophage adoptive transfer did not affect lesion number

In a gain of function experiment, the number and size of lesions formed in mice that had received adoptive transfer of large or small peritoneal macrophages into the peritoneal cavity at the time of endometrial tissue injection, was assessed. There was no difference in the number of lesions recovered from mice with had received adoptive transfer of large peritoneal macrophages, small peritoneal macrophages or no transfer (*figure 4.2A*). There was no significant difference in the size of lesions between groups however there was a modest reduction in the size of lesions from mice which had received adoptive transfer of large peritoneal macrophages (*figure 4.2B*).

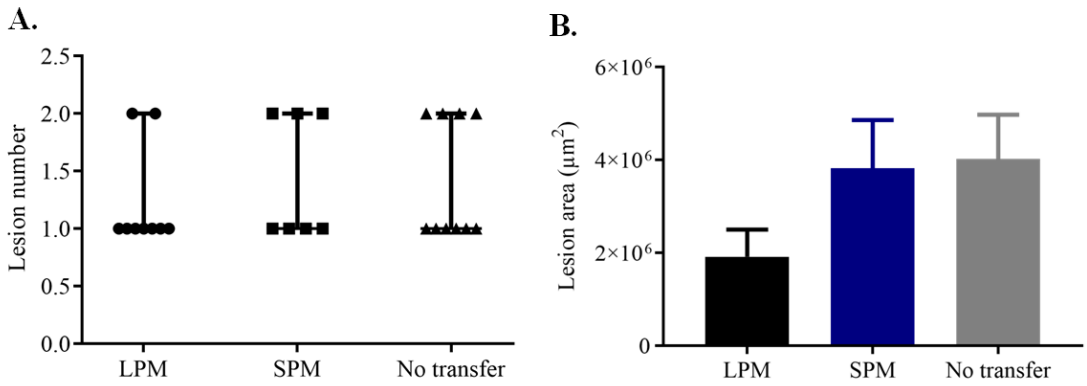


Figure 4.2. Adoptive transfer of small or large peritoneal macrophages into the peritoneal cavity of endometriosis mice did not significantly affect lesion number or sizeA: *Lesion number was similar from mice which had received adoptive transfer of LPM (n=9), SPM (n=7) or no transfer (n=10) 2 weeks after injection of endometrial tissue and cells. Data are expressed as medians with 95% confidence intervals.* ***B:*** *There was no statistical difference in lesion size from mice which had received adoptive transfer of LPM, SPM or no transfer, however we did observe a modest reduction in lesion size from mice which had received LPM. Data are expressed as median values in* ***A*** *and means ± SEM in* ***B****. Analysed by Analysis of variance (ANOVA).*

4.3.3 CCR2 expression is elevated in peritoneal and lesion resident macrophages in mice with endometriosis

Expression of CCR2 by F4/80+ mature macrophages in the peritoneal cavity of mice with endometriosis was analysed to begin to investigate the importance of CCR2 in macrophage recruitment to the peritoneal cavity in endometriosis. Peritoneal lavage fluid was isolated from naïve and sham mice and mice with induced endometriosis and expression of CCR2 analysed by flow cytometry. Mice with endometriosis had more significantly CCR2^{hi} F4/80^{hi} peritoneal macrophages compared to naive and sham mice (*figure 4.3A/B*).

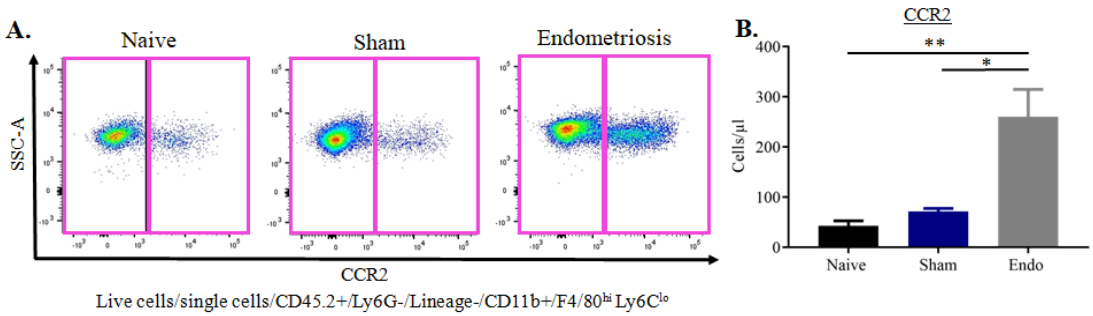


Figure 4.3. Mice with endometriosis had more CCR2+ peritoneal macrophages compared to sham and naive mice. A: *Flow plots showing CCR2+ F4/80^{hi} Ly6C^{lo} peritoneal macrophages in the peritoneal cavity of mice with endometriosis (n=5), naïve (n=4) and sham (n=4) animals 2 weeks after injection of endometrial tissue.* ***B:*** *Mice with endometriosis had significantly more CCR2+ peritoneal macrophages compared to naïve and sham animals. Data are expressed as means ± SEM. Analysis of variance (ANOVA), $^{*}P < 0.05$ and $^{**}P < 0.01$.*

Expression of CCR2 was also determined on endometriosis lesion resident macrophages (*figure 4.4A*). Endometriosis lesions from each mouse were pooled and analysed by flow cytometry. All lesion macrophages were CX3CR1+. A CD11b+ CX3CR1+ CCR2+ population was present which represented 53% (± SEM 5%) of CD11b+ CX3CR1+ cells as well as a CD11b+ CX3CR1+ CCR2- population (*figure 4.4B*). To assess the contribution of CCR2+ CD11b+ cells from donor endometrial tissue to lesions, CCR2 expression was analysed on CD11b+ cells in donor endometrial tissue from Csf1r-EGFP (MacGreen) mice (*figure 4.4C*). 70% of CD11b+ cells in donor endometrial tissue expressed CCR2 (*figure 4.4D*). Endometriosis lesions were subsequently analysed from WT mice, which had received MacGreen donor endometrial tissue, such that endometrial-derived

macrophages within lesions expressed GFP, allowing endometrial macrophages to be specifically identified in lesions. 100% of GFP+ CD11b+ cells within endometriosis lesions were also CCR2+ (*figure 4.4D*).

Taken together I have shown that CCR2 is elevated both in peritoneal cavity macrophages and macrophages in endometriosis lesions, identifying the potential for CCR2 to be important for macrophage chemotaxis in endometriosis.

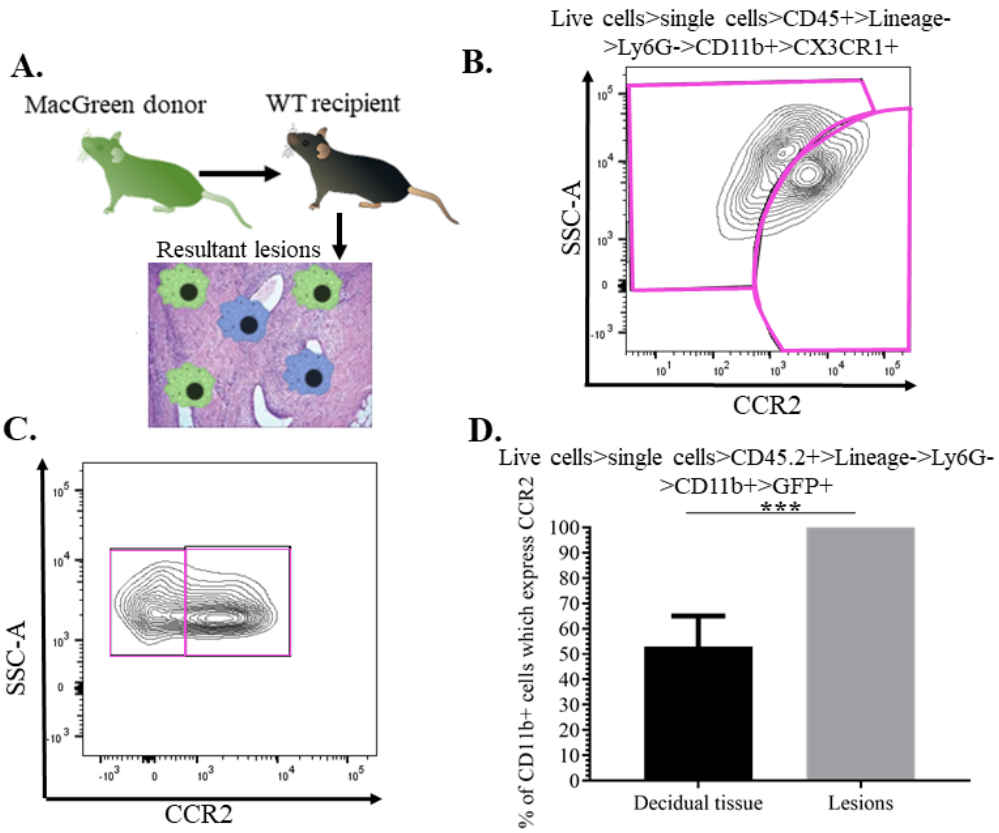


Figure 4.4. Lesion resident macrophages express the chemokine receptor CCR2. ***A:*** *Schematic demonstrating study design.* ***B:*** *Flow plot showing CCR2+, CD11b+, CX3CR1+ macrophages/monocytes in mouse endometriosis lesions 2 weeks after endometrial tissue injection, where all CD11b+ cells in the lesions were also CX3CR1+. 53% (± SEM 5%) of lesion resident CD11b+ CX3CR1+ cells expressed CCR2.* ***C:*** *Flow plot of CD11b+ macrophages/monocytes in donor decidualised endometrial tissue from Csf1r-EGFP mice showing expression of CCR2.* ***D:*** *Expression of CCR2 on endometrial GFP+ CD11b+ cells from Csf1r-EGFP donor endometrial tissue (n=6) and GFP+ endometrial-derived CD11b+ cells in endometriosis lesions from mice which had received Csf1r-EGFP donor endometrial tissue (n=6 animals where lesions were pooled from each animal) using flow cytometry. A lower proportion of macrophages expressed CCR2 in donor endometrial tissue compared with endometrial macrophages, which had persisted into endometriosis lesions (P=<0.001, student t test).*

4.3.4 CCR2 knockout mice develop endometriosis lesions and have an influx of inflammatory monocytes into the peritoneal cavity

In order to mechanistically determine the importance of CCR2 on endometriosis lesion development and monocyte/macrophage recruitment in disease, endometriosis was induced in CCR2 knockout mice alongside WT mice. Peritoneal immune cell populations were characterised in the peritoneal lavage fluid of CCR2 knockout mice with endometriosis, sham and naive mice as well as respective WT controls. Interestingly, CCR2 knockout mice with endometriosis had more CD45+ leukocytes in the peritoneal lavage fluid compared to sham and naive CCR2 knockout animals, indicating an influx of leukocytes into the peritoneal cavity in response to the presence of endometriosis lesions (*figure 4.5A*). Lineage+ cells (B cells, T cells, eosinophils, NK cells) were significantly increased in the peritoneal cavity of CCR2 knockout mice with endometriosis compared to WT mice with endometriosis, CCR2 knockout naive and sham mice (*figure 4.5B*). Interestingly, in this study WT mice with endometriosis had an increase in Ly6G+ peritoneal neutrophils compared to WT naive mice, however this increase was not observed in CCR2 knockout mice (*figure 4.5C*). Ly6C+ inflammatory monocyte numbers were not significantly increased in WT mice with endometriosis, although significant heterogeneity was observed across mice. CCR2 knockout mice however did have an increase in Ly6C+ peritoneal inflammatory monocytes compared to CCR2 knockout sham mice (*figure 4.5D*). There were no significant differences in Ly6C- F4/80+ mature macrophages or Ly6C+ F4/80+ macrophages transitioning from a monocyte to macrophage phenotype in the peritoneal cavity of any of the groups (*figure 4.5E/F*).

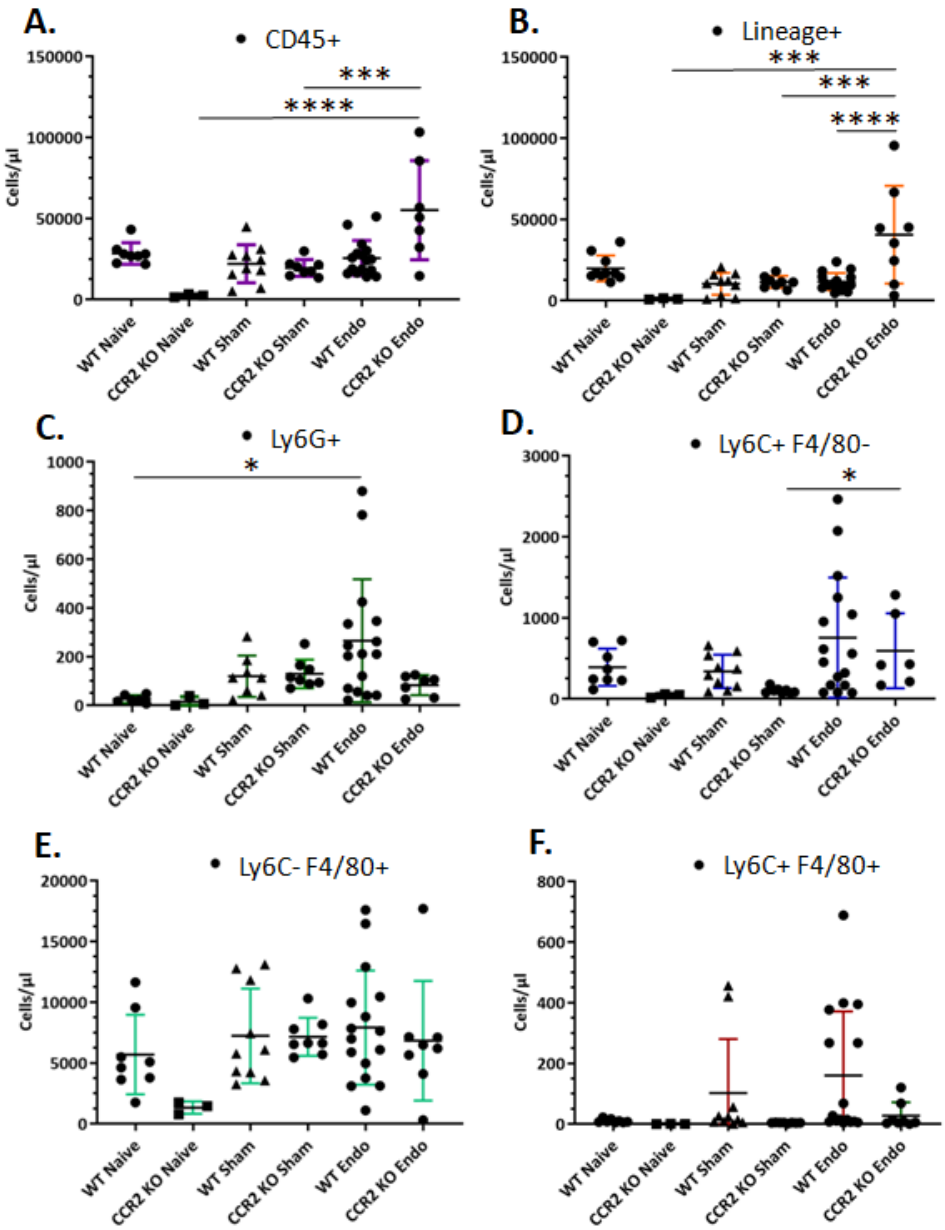


Figure 4.5. CCR2 knockout mice with endometriosis had an increase in Ly6C+ monocytes in the peritoneal cavity. ***A:*** *CD45+ leukocytes in the peritoneal cavity of WT animals with no treatment (n=8), CCR2 KO animals with no treatment (n=3), WT sham animals (n=10), CCR2 KO sham animals (n=8), WT mice with endometriosis (n=16) and CCR2 KO mice with endometriosis (n=8) were enumerated by flow cytometry. CCR2 KO mice with endometriosis had more CD45+ leukocytes compared to naive CCR2 KO mice and sham CCR2 KO mice.* ***B:*** *CCR2 KO mice had an increase in the lineage+ population in the peritoneal lavage fluid compared to naive CCR2 KO mice, sham CCR2 KO mice and WT mice with endometriosis. The lineage channel consisted of B cells, T cells, NK cells and eosinophils.* ***C:*** *WT mice with endometriosis had more Ly6G+ neutrophils in the peritoneal cavity compared to naive WT mice however CCR2 KO mice with endometriosis had similar neutrophil numbers to sham and naive CCR2 KO mice.* ***D:*** *CCR2 KO mice had significantly more Ly6C+ F4/80- monocytes in the peritoneal*

lavage fluid compared with CCR2 KO sham mice however WT mice with endometriosis had a similar number to sham. ***E:*** *There was no significant difference in Ly6C- F4/80+ macrophages across the groups.* ***F:*** *There was no significant difference in F4/80+ Ly6C+ cells across groups. Data are expressed as means ±SEM unless otherwise stated. Analysis of variance (ANOVA),* $^{*}P < 0.05$ *and* $^{**}P < 0.01$. **** P<0.001, **** P<0.0001.*

CCR2 knockout and WT mice with endometriosis developed a similar number of endometriosis lesions, which were a similar size *(figure 4.6A/B).*

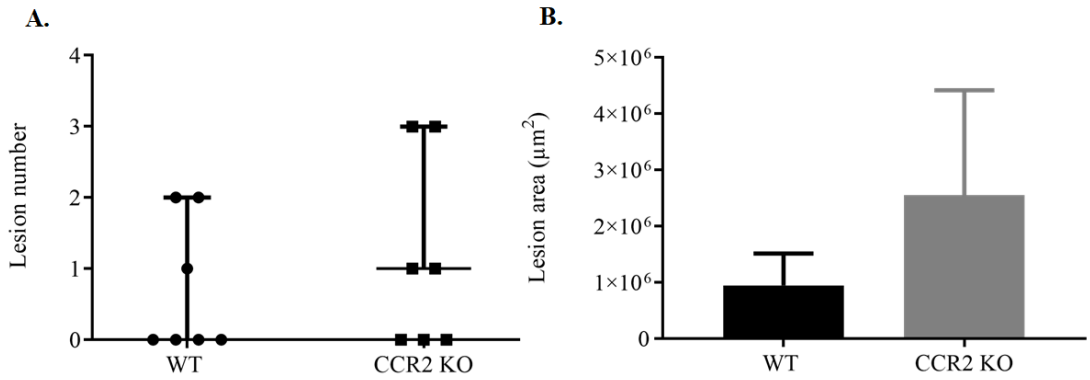


Figure 4.6. CCR2 knockout mice developed a similar number of lesions to WT *. **A:** The number of endometriosis lesions developed in WT and CCR2 KO mice. There was no difference in lesion number between WT (n=7) or CCR2 KO (n=7) mice with endometriosis at a 2 week time point. Data are expressed as medians with 95% confidence intervals.* ***B:*** *No difference in size of lesions was observed between WT and CCR2 KO mice with endometriosis. Data are expressed as median values in* ***A*** *and means ±SEM in* ***B****. Analysed by student t test.*

In order to assess the infiltration of Ly6C+ monocytes and F4/80+ macrophages into endometriosis lesions, dual immunofluorescence for Ly6C and F4/80 was performed on lesions from CCR2 knockout and WT mice (*figure 4.7A*). In CCR2 knockout lesions, Ly6C+ monocytes and F4/80+ macrophages were detected however a low abundance of Ly6C+ F4/80+ transitioning cells was observed (*figure 4.7A*). Compared to WT lesions, CCR2 knockout lesions had significantly less F4/80+ macrophages within lesion tissue (*figure 4.7A/B*). Interestingly, Ly6C+ monocytes were of similar abundance in CCR2 knockout and WT lesions with a trend for increased Ly6C+ monocytes in the CCR2 knockout lesions (*figure 4.7B*).

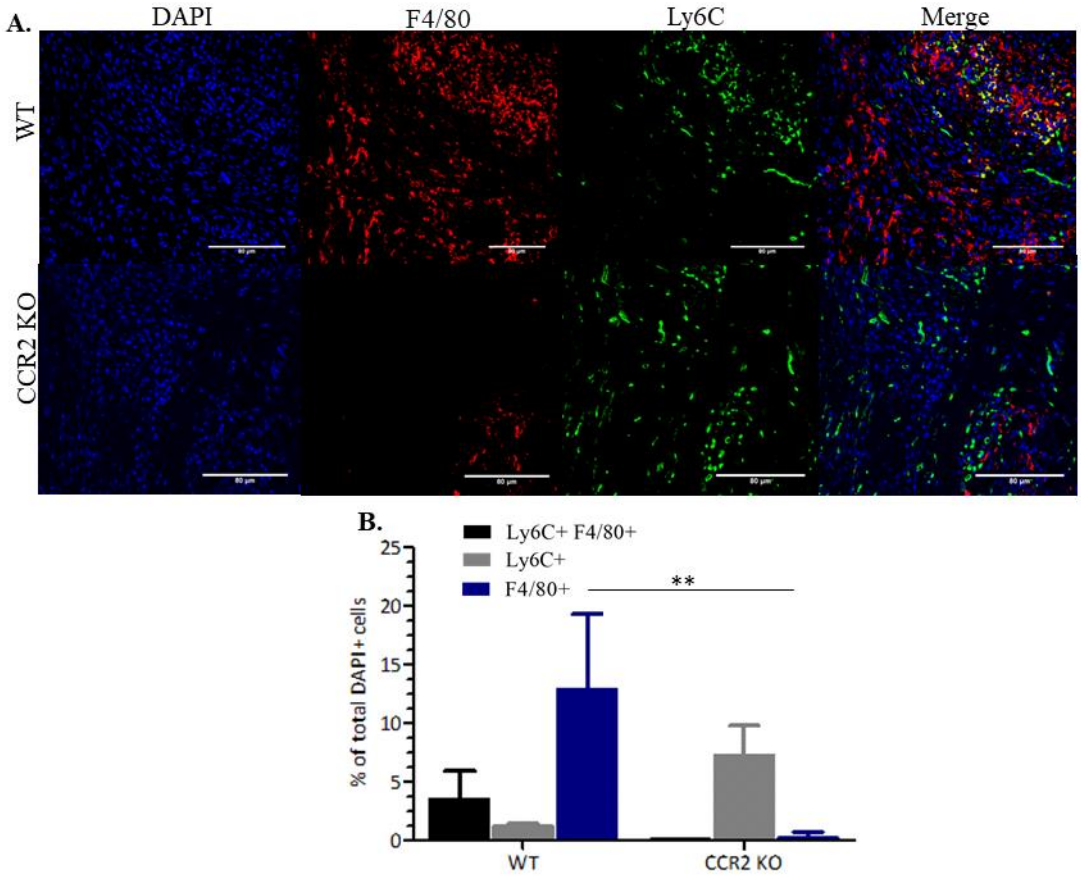


Figure 4.7. Endometriosis lesions from CCR2 KO mice had reduced lesion F4/80+ macrophages compared to WT but a similar number of Ly6C+ monocytes. A: *Ly6C F4/80 dual immunofluorescent staining on WT and CCR2 KO mouse endometriosis lesions.* ***B:*** *CCR2 KO lesions (n=3) had less F4/80+ cells than WT lesions (n=3) (P=0.0061). We also observed an absence of F4/80+ Ly6C+ cells in CCR2 KO lesions but an increase in Ly6C+ cells, although this was not significant. Data are expressed as median and range (due to low n number). Analysed by Mann-Whitney U test.*

4.3.5 CCL2 knockout mice develop more endometriosis lesions than WT and have increased CD45+ leukocytes in the peritoneal cavity

In order to investigate the role of the CCR2 receptor ligand, CCL2, on the development of endometriosis lesions, endometriosis was induced in CCL2 knockout alongside WT mice. Peritoneal immune cell populations were enumerated using flow cytometric analysis on peritoneal lavage fluid. CCL2 knockout mice with endometriosis and sham mice had an increase in CD45+ peritoneal leukocytes compared to WT endometriosis and sham mice respectively (*figure 4.8A*). CCL2 knockout mice also had an increase in lineage+ cells (B cells, T cells, eosinophils,

NK cells) (*figure 4.8B*). There were no significant increases in Ly6G+ neutrophils across the groups however both WT and CCL2 knockout mice with endometriosis exhibited heterogeneity (*figure 4.8C*). Ly6C+ monocyte numbers were similar across the groups however some WT and CCL2 knockout mice with endometriosis demonstrated an increase in this population, but again this was heterogeneous (*figure 4.8D*). F4/80+ mature macrophage numbers were similar across the groups (*figure 4.8E*). Ly6C+ F4/80+ transitioning cell numbers were similar across groups (*figure 4.8F*). Notably, CCL2 knockout sham animals had homogeneously low numbers of Ly6C+ F4/80+ cells however CCL2 knockout mice with endometriosis had a heterogeneous increase in this population (*figure 4.8F*).

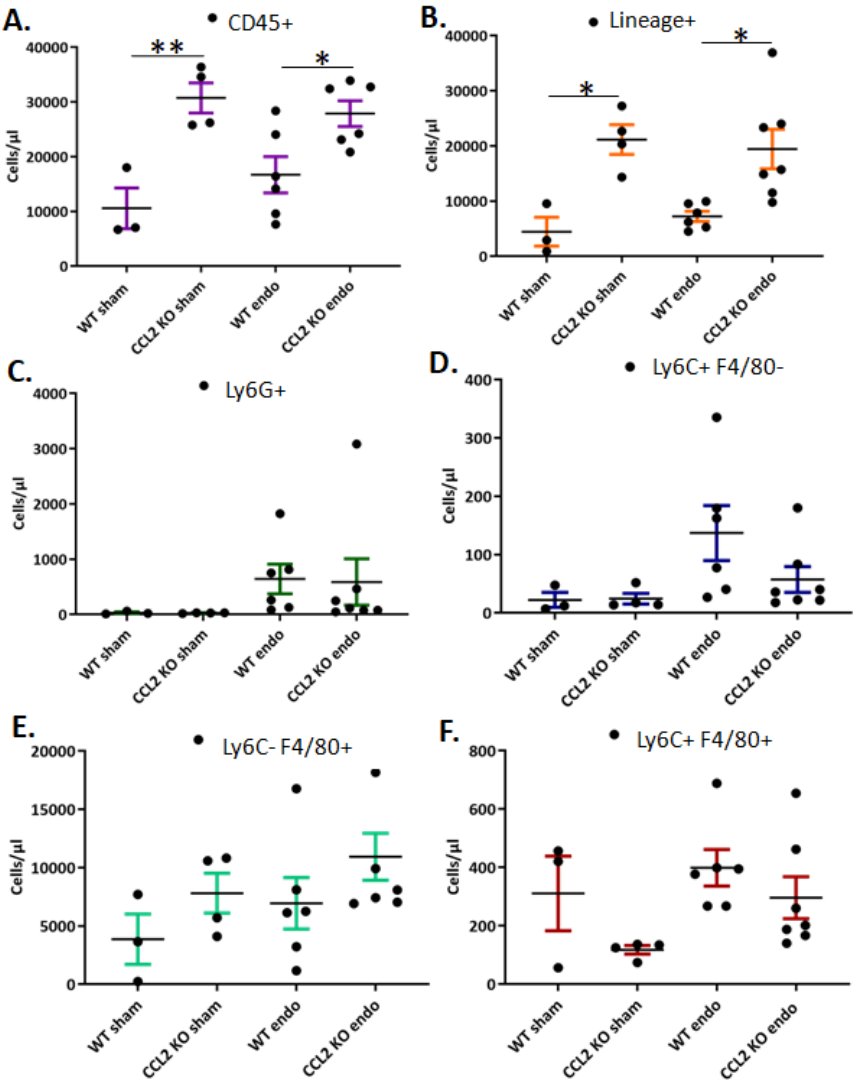


Figure 4.8. CCL2 knockout sham and endometriosis mice had an increase in peritoneal CD45+ leukocytes. A: *CD45+ leukocytes in the peritoneal lavage fluid of WT sham animals (n=3), CCL2 KO sham animals (n=4), WT endometriosis mice (n=6) and CCL2 KO mice with endometriosis (n=7) were enumerated by flow cytometry. CCL2 KO sham animals had significantly more CD45+ leukocytes compared with WT sham controls. CCL2 KO mice with endometriosis also demonstrated a significant increase in CD45+ leukocytes compared to WT mice with endometriosis.* ***B:*** *Sham and endometriosis CCL2 KO mice had an increase in the lineage+ population in the peritoneal lavage fluid compared to WT sham and endometriosis mice respectively. The lineage channel consisted of B cells, T cells, NK cells and eosinophils.* ***C:*** *There were no significant changes in $Ly6G^+$ neutrophil numbers in the peritoneal lavage fluid, however we did observe a modest increase in the $Ly6G^+$ population in WT and CCL2 KO mice with endometriosis compared to the respective sham controls, however this was heterogeneous.* ***D:*** *There was no significant difference in Ly6C+ F4/80- monocyte numbers between groups. We did however observe increased monocyte numbers in mice with endometriosis compared to sham controls and this was seen in both WT and CCL2 KO mice.* ***E:*** *There was no difference in Ly6C- F4/80+ mature macrophage numbers across the groups.* ***F:***

There was no significant difference in Ly6C+ F4/80+ cells across the groups however we did observe a modest increase in this population between CCL2 KO sham and endometriosis mice. Data are expressed as means ±SEM unless otherwise stated. Analysis of variance (ANOVA), $^{*}P < 0.05$ and $^{**}P < 0.01$.

CCL2 knockout mice with endometriosis developed more endometriosis lesions than WT mice (*figure 4.9A*). Endometriosis lesions formed in CCL2 knockout and WT mice were a similar size (*figure 4.9B*).

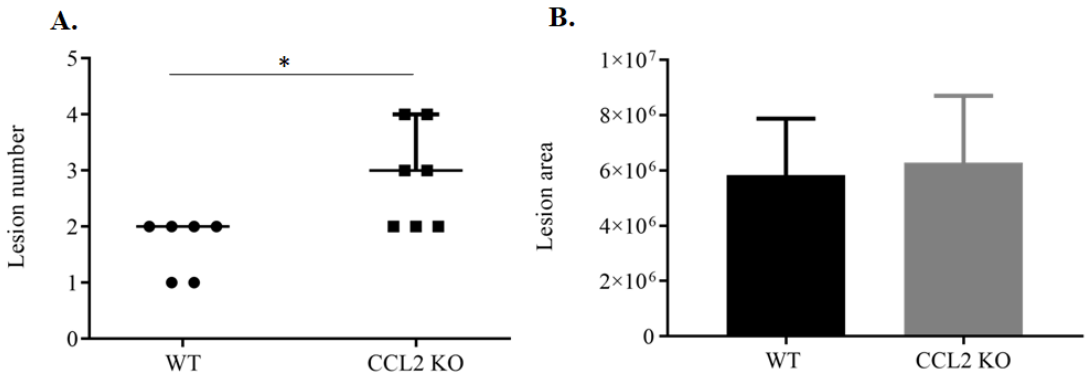


Figure 4.9. CCL2 KO mice developed more endometriosis lesions than WT mice. ***A:*** *CCL2 KO mice developed significantly more endometriosis lesions compared to WT animals at a 2 week time point (P=0.0157). Data are expressed as medians with 95% confidence intervals.* ***B:*** *No difference was observed in the size of lesions between CCL2 KO and WT mice. Data are expressed as means ± SEM. Analysed by student t test.*

Infiltration of Ly6C+ monocytes and F4/80+ macrophages into CCL2 knockout and WT endometriosis lesions was investigated using dual immunofluorescence for Ly6C and F4/80 (*figure 4.10A*). Both CCL2 knockout and WT endometriosis lesions were infiltrated by Ly6C+ monocytes, Ly6C+ F4/80+ transitioning cells and F4/80+ macrophages. The number of these cell populations in CCL2 knockout and WT endometriosis lesions was similar (*figure 4.10B*).

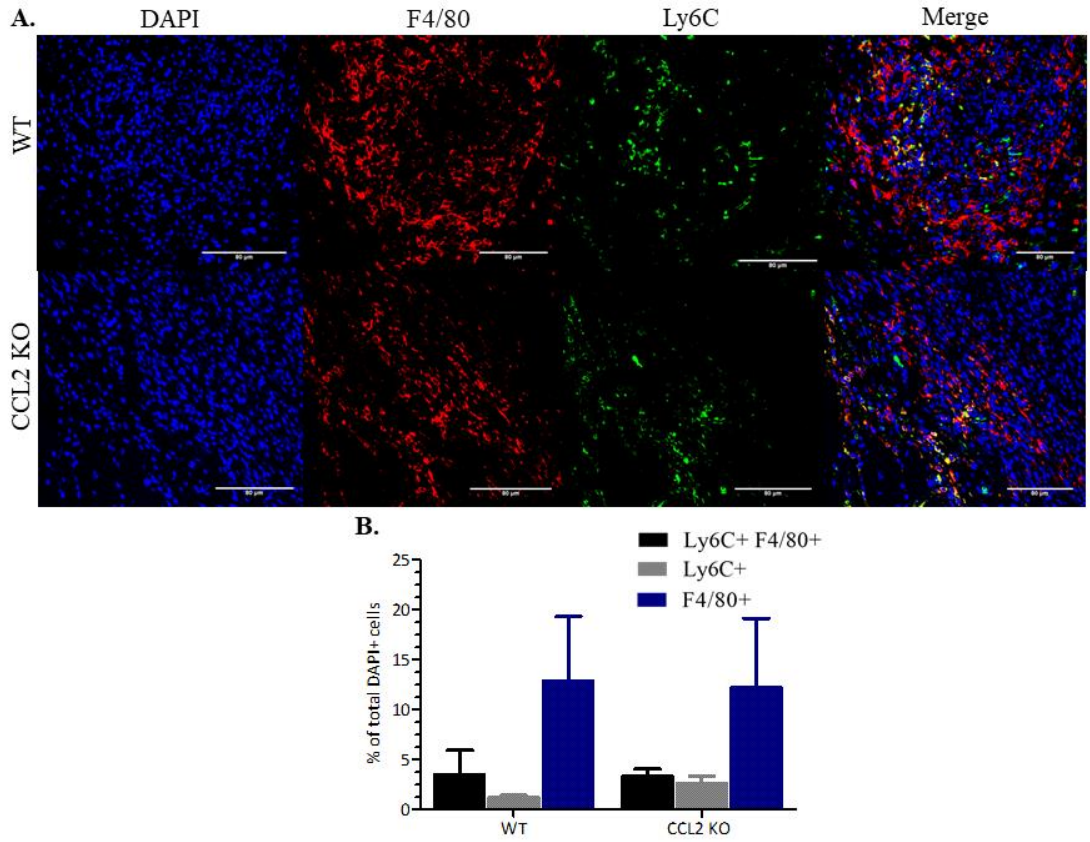


Figure 4.10. Endometriosis lesions from CCL2 KO and WT mice had a similar number of Ly6C+, F4/80+ and Ly6C+ F4/80+ cells. A: *Ly6C F4/80 dual immunofluorescent staining on WT and CCL2 KO mouse endometriosis lesions.* ***B:*** *CCL2 KO lesions (n=6) had a similar number of F4/80+, Ly6C+ and Ly6C+ F4/80+ cells compared to WT lesions (n=3). Data are expressed as median and range. Data analysed by Mann-Whitney U test.*

4.3 Summary

In this chapter I used macrophage depletion, adoptive transfer and blocking of a key monocyte recruitment axis in our mouse model of induced endometriosis in order to investigate the role of macrophages and monocytes of different origins. I depleted macrophages in donor endometrial tissue but found no significant effect on subsequent lesion number and size. I also characterised lesion number and size in mice, which had received adoptive transfer of large or small peritoneal macrophages and found no differences. I demonstrated CCR2 is expressed on a large proportion of peritoneal and lesion-resident macrophages. CCR2 knockout mice develop an equal number of endometriosis lesions to wild type controls but have an influx of Ly6C+ monocytes into the peritoneal cavity which infiltrate lesions, however F4/80+ macrophages in lesions are reduced. CCL2 knockout mice with endometriosis have a

similar number of peritoneal Ly6C+ monocytes and F4/80+ macrophages as WT controls and endometriosis lesions from CCL2 knockout mice exhibit no alterations in numbers of Ly6C+ monocytes and F4/80+ macrophages, identifying that recruitment of these populations in disease can occur independent of the CCR2-CCL2 signalling pathway.

4.4 Discussion

In this chapter I investigated the role of the CCR2-CCL2 chemokine-signalling axis in the recruitment of Ly6C^{hi} monocytes and F4/80+ macrophages to endometriosis lesions. In a study using a mouse model of pancreatic ductal adenocarcinoma, induction of disease in CCR2 knockout mice was able to significantly reduce the recruitment of MHC II+ monocyte-derived tumour associated macrophages (Zhu *et al.*, 2017). I was able to demonstrate that endometriosis lesions from CCR2 knockout mice had reduced F4/80+ macrophage numbers, indicating that similarly CCR2 may be required for recruitment to lesions. Conversely, CCR2 knockout mice with endometriosis had a significant influx of Ly6C^{hi} monocytes into the peritoneal cavity, which was not observed in sham controls. This indicates that in the absence of CCR2, Ly6C^{hi} monocytes still receive a chemotactic signal causing their mobilisation from the bone marrow and subsequent extravasation into the peritoneal cavity in the endometriosis disease state. It has been demonstrated however that CCR2 is a promiscuous receptor that can bind to other chemokines such as CCL8, CCL13 and CCL7 (Lira and Furtado, 2012). In order to determine whether the effects I was seeing were actually due to the CCL2 ligand, I utilised CCL2 knockout mice. CCL2 knockout mice had comparable peritoneal cavity Ly6C^{hi} monocyte numbers to WT mice with endometriosis, suggesting that Ly6C^{hi} monocyte extravasation into the peritoneal cavity was not inhibited even in the absence of CCL2 in endometriosis. However, to gain a statistically significant difference in Ly6C^{hi} peritoneal monocyte number between WT and CCL2 knockout animals, I would need 7 more mice in each group (power calculation performed using www.stat.ubc.ca/). This fairly small increase in N number suggests that there may have been a difference in Ly6C^{hi} peritoneal monocytes between the WT and CCL2

KO mice, but that the sample size is too small to detect this. Thus, this may be worth repeating in future studies.

Notably, CCR2 and CCL2 knockout mice with endometriosis both had increased peritoneal CD45+ leukocytes compared to WT controls, and this was partly attributed to an increase in the lineage+ population which consisted of T cells, B cells, NK cells and eosinophils. This confounding factor makes it difficult to conclude whether outcomes of the experiments such as lesion number were due to the absence of the CCL2 ligand or the increase in these cell populations. It is difficult to infer as to the mechanism behind this increase as I cannot differentiate which cell type the increase in CD45+ leukocytes was derived from due to the fact that the lineage cell markers were all conjugated to the same fluorochrome. The increased number of lesions in CCL2 knockout animals therefore may be attributed to modulation of other immune cell populations in our mouse model due to the absence of CCL2. For example, cytotoxic activity as well as chemotaxis of NK cells has been shown to be enhanced by CCL2 (Yoneda *et al.*, 2000; Inngjerdingen, Damaj and Maghazachi, 2001), and we know that a reduction in NK cell cytotoxic activity is present in the peritoneal cavity of women with endometriosis, and this is thought to stimulate disease (Jeung, Cheon and Kim, 2016). It could be therefore that the increase in lesion number in CCL2 knockout mice is a result of modulation of the function and number of immune cells in the peritoneal cavity, and this may not be specific to monocytes/macrophages. It is thus difficult to conclude, with the current granularity of data, the mechanism behind increased lesion number of CCL2 knockout animals, which have normal numbers of peritoneal and lesion Ly6C^{hi} monocytes and F4/80+ macrophages.

CCR2 knockout animals had a similar number of lesions to WT, despite a reduction in F4/80+ lesion macrophages. It could be that differential macrophage populations exist within endometriosis lesions that play distinct roles in lesion development, a question I begin to investigate in chapter 5. For example, in pancreatic ductal adenocarcinoma, recruited monocyte-derived macrophages are important for antigen presentation in the tumour and depletion of this population does not result in a change in tumour growth (Zhu *et al.,* 2017). Thus, monocyte-derived macrophages

fulfil a function within the tumour microenvironment but this is not critical to the growth of the tumours. I cannot conclude from my data exactly which subset of macrophages was depleted in endometriosis lesions from CCR2 knockout animals, however the concept that macrophages with differential roles in disease exist within the lesion environment could explain why I didn't see a difference in lesion size.

Overall, this data provides the first evidence that Ly6C^{hi} monocytes in endometriosis are recruited from the bone marrow where they eventually become incorporated into endometriosis lesions. Importantly, I have demonstrated that this recruitment can occur independent of CCR2/CCL2. Whether there is a compensation effect from other chemokines when CCR2 or CCL2 signalling is interrupted is unknown, for example we also know that CCL5 is up-regulated in the peritoneal cavity of mice with endometriosis (Greaves *et al.*, 2014) and that in mice CCL5 can mediate the chemotaxis of monocytes in steady state (Raghu *et al.*, 2017). However, the chemokine pathway responsible for the recruitment of Ly6C^{hi} monocytes in the absence of CCR2 or CCL2 is un-defined. Whilst the CCR2/CCL2 chemokine pathway may be important for recruitment of Ly6C+ monocytes in disease, this pathway appears to be redundant.

Conversely, I did observe that endometriosis lesions from CCR2 knockout mice had a reduced number of F4/80+ macrophages. The fact monocyte number was not affected but macrophage number was reduced, suggests that this reduction in F4/80+ macrophages is derived from a population other than the monocyte-derived macrophages (as these are derived from Ly6C+ monocytes). In this chapter I demonstrated that mice with endometriosis have increased CCR2+ F4/80+ macrophages in the peritoneal cavity and that 53% of lesion macrophages expressed CCR2. It is possible therefore that this population of macrophages relies on CCR2 for chemotaxis to endometriosis lesions, and thus, when CCR2 is abrogated, this recruitment is blocked. I cannot definitively say whether this population of CCR2+ peritoneal macrophages do become incorporated into endometriosis lesions, as I did not perform lineage tracing studies to interrogate this, however the fact that half of the lesion macrophages express CCR2 supports the notion that CCR2+ macrophages are recruited to lesions. It could be, therefore, that whilst CCR2 is redundant in

endometriosis for the recruitment of Ly6C^{hi} monocytes, CCR2 is in fact elemental to the recruitment of a subset of F4/80+ macrophages to endometriosis lesions. Hence, inhibiting CCR2 is able to block F4/80+ macrophage accumulation in lesions. However, in endometriosis lesions from CCL2 knockout animals, I saw a similar number of F4/80+ macrophages to WT. We know that CCR2 can bind to other chemokines such as CCL8, CCL13 and CCL7, which can induce macrophage recruitment (Lira and Furtado, 2012). Thus, by knocking out CCL2, there may still be interaction of chemokines with CCR2 which are able to induce macrophage chemotaxis. As I characterised that a large proportion of lesion macrophages express CCR2, it is possible that CCL2 signalling is not fundamental to macrophage recruitment to lesions but that chemokine signalling via CCR2 is indeed responsible for this recruitment, be this through CCL2, CCL8, CCL13 or CCL7 (Lira and Furtado, 2012). Whether CCL2 is usually required for this recruitment and compensation from other chemokines occurs in its absence, is unclear from my data. I would hypothesise however that this is the case, due to the fact that CCR2-CCL2 signalling is the classical pathway for monocyte/macrophage recruitment in steady state and in a number of cancers (Qian *et al.*, 2011; Noy and Pollard, 2014; Lim *et al.*, 2016).

By disrupting the CCR2-CCL axis I was aiming to prevent monocytes from being recruited to lesions, however I did not achieve this due to apparent redundancy in this pathway. Thus, the role of Ly6C^{hi} monocytes in endometriosis pathophysiology remains undefined, as to my knowledge, no other studies have investigated this.

In this chapter I also aimed to discern the effect of endometrial macrophage depletion on the development of endometriosis lesions in our model. Whilst depletion was achieved (34% of the F4/80^{hi} Ly6C^{lo} macrophages and 32% of the Ly6C^{hi} monocytes), this was not significantly different to WT donor endometrial tissue. Due to the fact that macrophages are thought to be important for the decidualisation process in the mouse model of menstruation (Thiruchelvam *et al.*, 2013; Cousins *et al.*, 2016), depletion of endometrial macrophage populations in donor mice is therefore a play off between achieving decidualisation and depleting macrophages to exert an effect on the development of lesions. In my studies, I was able to achieve

decidualisation in all of the mice whilst also depleting macrophages by dosing doxycycline for 4 days (days 15-19). To achieve significant macrophage depletion, doxycycline could be dosed for a longer time period, but it must be taken into account that this has the potential to disrupt the generation of donor decidualised endometrial tissue. In order to gain statistical significance in lesion number with the macrophage depletion I achieved in my studies, I would need to use 33 mice for each group (power calculation performed using www.stat.ubc.ca). Due to time limits and limited access to the CSF1R cKO mouse line, I was not able to repeat my studies.

Next, I analysed the number and size of lesions from mice which had received adoptive transfer of either LPM or SPM, and thus had an increased number of either LPM or SPM within the peritoneal cavity. This data did not produce any significant differences. To gain a significant difference in lesion number between mice with no transfer and those which had received adoptive transfer of SPM, power calculations suggest that an infinite number of mice would need to be used (hence, there was no observable difference). To observe a significant difference in lesion number between mice with no transfer and mice which received transfer of LPM, 393 mice would need to be used for each group (power calculation performed using www.stat.ubc.ca). Thus, it appears highly unlikely that the transfer of LPM into the peritoneal cavity in my studies would be able to exert a biological effect in lesion development. This could be due to the number of cells transferred into the peritoneal cavity, as it is difficult to predict whether this is enough to exert a biological effect. The transfer of more LPM may address this issue, or a more definitive method to decipher the roles of LPM and SPM in endometriosis lesion development may be through depletion studies. Clodronate liposome depletion of F4/80^{hi} and F4/80^{hi} Ly6C^{hi} peritoneal macrophages is able to decrease the number of mice which develop lesions in our mouse model (13 out of 14 developed lesions for non-depleted and 9 out of 17 for depleted) (Forster *et al*., 2019). It would be interesting therefore to discern in which peritoneal macrophage compartment this effect on lesion number is attributed to. Due to the fact that LPM but not SPM infiltrate lesions (data from chapter 3), I would hypothesise that LPM but not SPM play roles in endometriosis lesion development in our model. The fact that LPM are associated with more homeostatic, tissue reparative roles and SPM are involved in stimulating

inflammation and clearing foreign material, also supports this notion (Bain and Jenkins, 2018). Further research is thus required to discern the role of LPM and SPM in the development of endometriosis lesions.

Chapter 5 - Lesion resident macrophages exhibit phenotypic heterogeneity

5.1 Introduction

Current therapies which target macrophages, largely in cancer studies, have relied upon the targeting of generic macrophage markers which are expressed by macrophage populations throughout the body (Butowski *et al.*, 2016; Gomez-Roca *et al.*, 2019). However, recent studies have indicated that macrophage populations within disease states are often heterogeneous, and that differential targeting of specific macrophage populations may provide clinical benefit without having significant off-target effects (Noy and Pollard, 2014). Deciphering what makes disease-associated macrophages 'unique' compared to healthy tissue macrophage populations is therefore an important research area. In endometriosis, pathological, disease-associated macrophages remain poorly characterised.

In cancers, we know that macrophages become 'educated' by the tumour microenvironment to adopt pro-disease characteristics and a unique genetic signature (Cassetta *et al.*, 2019). In endometriosis, there have been no transcriptomic studies on lesion macrophages and thus our understanding of macrophage education within endometriosis lesions is minimal. One paper by Johan *et al* investigated macrophage phenotype after incorporation in endometriosis lesions, using a mouse model where human endometrial tissue was grafted into immunodeficient mice harbouring a macrophage specific GFP gene. After incorporation into lesions, GFP+ macrophages became more 'M2-like', or anti-inflammatory, over time (Johan *et al.*, 2019b). A limitation of this study is that only four markers were investigated by immunodetection to define macrophage phenotype: iNOS and MHC II for inflammatory macrophages, and arginase 1 and CD204 for anti-inflammatory macrophages. It is likely therefore that this data does not reflect the true complexities of macrophage phenotype within lesions and does not take into account that there may be multiple macrophage populations present. What this data does infer, however, is that macrophages do appear to become 'educated' by the lesion microenvironment.

In order to characterise macrophage populations associated with disease, researchers commonly turn to sequencing techniques, which introduce less bias and achieve novel insight into the complexities of macrophage biology. Recently, single cell

RNA sequencing has been increasingly utilised to answer questions relating to macrophage heterogeneity in disease. Single cell RNA sequencing relies on nucleotide barcodes, which attached to the mRNA in each individual cell, such that mRNA transcripts can be evaluated on a cell-by-cell basis. Recently, single cell RNA sequencing has been used to define monocyte and macrophage populations in atherosclerosis and revealed novel macrophage transcriptomic signatures which are associated with varying stages of disease (Lin *et al.*, 2019). Single cell studies in cancer have also given insight into macrophage dynamics within the tumour environment (Cassetta *et al.*, 2019). Cassetta *et al* demonstrated that TAMs from women with endometrial or breast cancer clustered separately using principal component analysis. Gene ontology testing identified up-regulated genes involved in processes such as phagocytosis, immune response, cell communication, and blood vessel development, identifying a divergence in gene expression of macrophages which had been 'educated' by the tumours (Cassetta *et al.*, 2019). In this chapter, I utilise single cell RNA sequencing to reveal novel insights into macrophage heterogeneity in endometriosis lesions.

5.2 Aims and hypotheses

I hypothesised that endometriosis lesion resident macrophages are a heterogeneous population that have a transcriptional profile which is unique from eutopic endometrial macrophages or macrophages from the peritoneal cavity.

The aims of this chapter were to:

1. Characterise the transcriptional profiles of endometriosis lesion macrophages compared to eutopic endometrial macrophages and peritoneal macrophages in our mouse model of induced endometriosis
2. Interrogate endometriosis lesion resident macrophage phenotype heterogeneity at the protein and RNA level
3. Determine whether endometriosis lesion resident macrophages have the capacity to expand via *in situ* proliferation

5.3 Results

5.3.1 Lesion resident macrophages are a heterogeneous population which exhibit differential expression of CCR2 and MHC II

In order to initially determine whether lesion resident macrophages can be divided into sub-populations based on surface marker expression, flow cytometry was utilised to interrogate endometriosis macrophages within lesion tissue. Macrophages were analysed for expression of activation markers that are indicative of macrophage phenotype either as an anti-inflammatory phenotype (CX3CR1) or a pro-inflammatory phenotype (CCR2, CD86, MHC II). To gain further insight into macrophage phenotype dynamics, fate mapping studies were performed which allowed the identification of endometrial-derived macrophage and recruited macrophage phenotype within endometriosis lesions.

Initially, CD11b+ macrophage/monocyte populations were interrogated in the decidualised eutopic endometrium. Surface expression of CX3CR1, CCR2, MHC II and CD86 was analysed (*figure 5.1A*). Using these markers, eight sub-populations were identified based on differential expression of the selected markers . All endometrial CD11b+ cells were CX3CR1^{hi} (*figure 5.1B*). The largest population, which accounted for 26% (± SEM 2.5%) of cells, was CD11b+, CX3CR1^{hi}, CCR2^{hi}, MHC II^{lo} and CD86^{lo} (*figure 5.1B*). The tight variance across mice in the macrophage populations identified highlighted the consistency in macrophage phenotypic heterogeneity within the tissue (*figure 5.1B*). To determine the phenotypic heterogeneity of endometrial macrophages that had become incorporated into endometriosis lesions, GFP+ CD11b+ cells were analysed in endometriosis lesions from WT mice that had received intraperitoneal injection of MacGreen donor endometrial tissue. Of the GFP+ CD11b+ endometrial macrophages incorporated into lesions a single population was delineated which was CX3CR1^{hi}, CCR2^{hi}, MHC II^{lo} and CD86^{hi} (*figure 5.1C/E*), suggesting phenotypic heterogeneity evident in endometrial macrophages is lost upon incorporation into lesions.

Next, the phenotype of GFP-, recipient derived CD11b+ cells were analysed in endometriosis lesions (*figure 5.1D*). A total of four populations were identified based on differential expression of CCR2 and MHC II in the GFP- CD11b+ recipient

derived population, the largest population of which had the phenotype CX3CR1^{hi}, CCR2^{hi}, MHC II^{lo} and CD86^{hi}. Notably, this population had the same phenotype as the GFP+ endometrial-derived macrophages within endometriosis lesions (*figure 5.1F*).

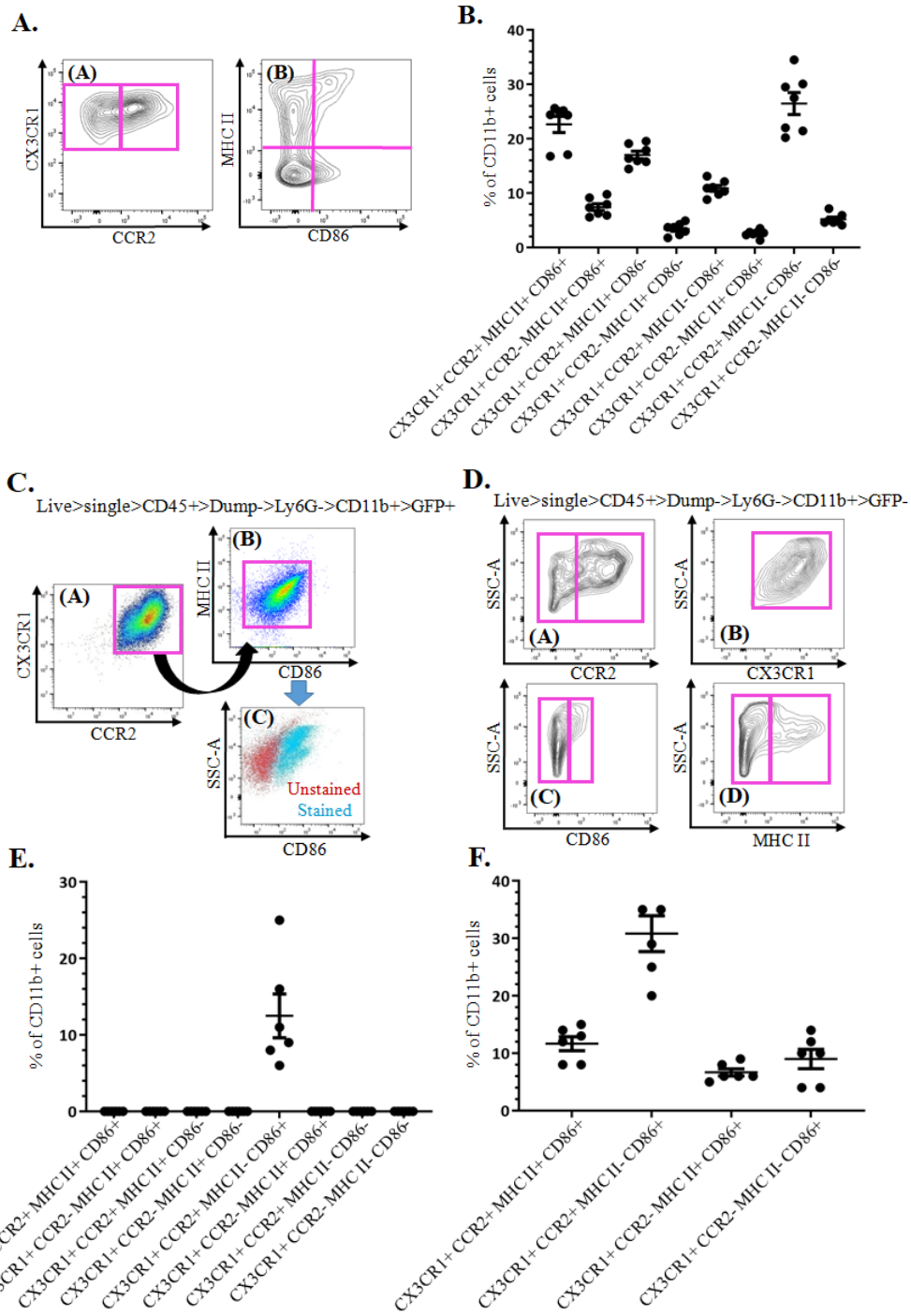


Figure 5.1. Lesion resident macrophages are a heterogeneous population which exhibit differential expression of CCR2 and MHC II. A: *Flow plots of CD11b+ cells in donor endometrial tissue showing expression of CX3CR1, CCR2, MHC II*

and CD86. ***(A)*** *All CD11b+ cells were CX3CR1^{hi}of which there was both a CCR2^{hi} and CCR2^{lo} population (hi and lo expression is denoted as + and - respectively on graphs).* ***(B)*** *A major population of CD11b+ MHC II^{lo} CD86^{lo} cells predominated in the tissue however MHC II^{hi} CD86^{hi}, MHC II^{hi} CD86^{lo} and MHC II^{lo} CD86^{hi} populations were also present.* ***B:****Eight populations of CD11b+ cells were identified in donor endometrial tissue based on expression of CX3CR1, CCR2, MHC II and CD86 (n=7 mice).* ***C:*** *Flow plots of GFP+ endometrial-derived CD11b+ cells in endometriosis lesions where mice had received Csf1r-EGFP donor endometrial tissue.* ***(A)*** *Endometrial-derived CD11b+ cells in lesions were CCR2^{hi} CX3CR1^{hi}.* ***(B)*** *Endometrial-derived lesion CD11b+ cells were MHC II^{lo} CD86^{hi}.* ***(C)*** *Unstained GFP+ CD11b+ lesion cells with or without incubation with a CD86 antibody, demonstrating an increase in CD86 expression in the stained population.* ***D:*** *Flow plots of GFP- recipient derived CD11b+ cells in the endometriosis lesions of mice which had received Csf1r-EGFP donor endometrial tissue.* ***(A)*** *Both CCR2^{hi} and CCR2^{lo} populations were present in the GFP- CD11b+ population.* ***(B)*** *All GFP- CD11b+ cells were CX3CR1^{hi}.* ***(C)*** *Both CD86^{hi} and CD86^{lo} recipient derived CD11b+ populations were present.* ***(D)*** *Both CD11b+ MHC II^{hi} and CD11b+ MHC II^{lo} populations were present.* ***E:*** *One population of GFP+ endometrial derived CD11b+ cells was identified in mouse endometriosis lesions which was CX3CR1^{hi} CCR2^{hi} MHC II^{lo} CD86^{hi}(n=6 mice).* ***F:*** *Four populations of CD11b+ cells were identified in the GFP- recipient derived macrophage population in lesions based on expression of CX3CR1, CCR2, MHC II and CD86 (n=6 mice). The predominant population was CX3CR1^{hi} CCR2^{hi} MHC II^{lo} CD86^{hi}. Data are shown as mean ±SEM.*

5.3.2 Endometriosis lesion resident macrophages are transcriptionally unique from eutopic endometrial and peritoneal macrophages

Once I had determined that endometriosis lesion resident macrophages exhibit phenotypic heterogeneity and can be classed into sub-populations based on cell surface marker expression (*figure 5.1*), I wanted to assess phenotypic heterogeneity using a single cell RNA-Seq which introduces less bias into data collection (where the markers are not pre-defined) and gives greater granularity into the true heterogeneity of macrophages in a sample. Unbiased transcriptional profiling of CD45+ cells in lesions (6006 cells), donor endometrial tissue (1306 cells) and peritoneal lavage cells from sham (5645 cells) and endometriosis mice (6720 cells) was performed by Edinburgh Genomics, such that I could compare gene expression between the tissues and characterise macrophage heterogeneity within endometriosis lesions.

Using Loupe Cell Browser 3.0.1 software I visualised the samples using t-distributed stochastic neighbour embedding (tSNE) plots. In these plots, each cell is represented

as a dot on the graph. The cells are distributed according to their transcriptomic signature, such that cells with a more similar transcriptomic signature can be found closer together on the graph and thus inferences can be made based on the clustering of cells on the tSNE plot. For this thesis, graph-based clustering was visualized using Loupe Cell Browser 3.0.1 software. Cell clusters produced using graph-based clustering were analysed for restricted or increased expression of the most highly up-regulated genes. In some cases, the most highly up-regulated genes in a cluster did not exhibit restricted or increased expression to the cluster in question, suggesting that this did not represent a biologically distinct population (the sample may have been 'over-clustered'). In this manner, the sample was re-analysed such that only 'true' clusters with up-regulated genes which could define or were up-regulated in the specified group of cells were taken forward for analysis.

Figure 5.2 shows tSNE plots of all of the samples clustered together based on library id in the aggregate tSNE plot can be visualised. In the samples, CD45+ leukocytes were defined based on expression of pre-defined markers expressed by specific leukocyte populations. Macrophages were defined based on expression of *Csf1r*, *Adgre1* (F4/80) and *Itgam* (CD11b). B cells were defined based on expression of *Cd19*, *Cd79a* and *Ly6d*. NK and T cells clustered together and were defined based on expression of *Gzmc*, *Gzme*, *Gzmf*, *Nkg7* and *Thy1* (NK cells) and *Cd3g*, *Cd3d*, *Ccl5*, *Il2rb* (T cells). Notably, *Ly6g+* neutrophils were absent from the samples and it has been previously noted by other researchers that neutrophils are difficult to sequence on the 10X Genomics system (data unpublished). Macrophages were the predominant cell type in all of the samples (*figure 5.2B*). Notably, macrophages in the endometriosis lesion samples demonstrated significant overlap with eutopic endometrial macrophages (macrophages from the donor endometrial tissue), suggesting that these macrophage populations are transcriptionally similar when compared with peritoneal macrophages. This area of overlap is indicated with a 1 in *figure 5.2B.* A population of macrophages from the endometriosis lesion sample also clustered with the peritoneal macrophages, suggesting that this population is transcriptionally similar to the peritoneal macrophages. This area is indicated with a 2 in *figure 5.2B.* The fact that endometriosis lesion macrophages cluster with both

the endometrial and peritoneal macrophages is consistent with the data presented in chapter 3, which demonstrated that endometriosis lesion resident macrophages are derived from both endometrial and peritoneal macrophages (as well as monocyte-derived macrophages).

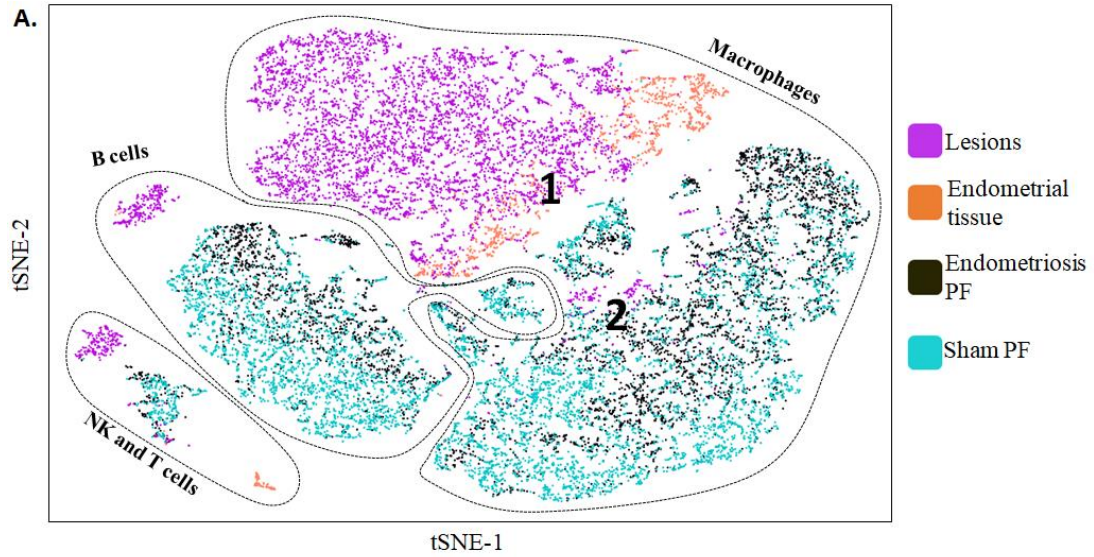
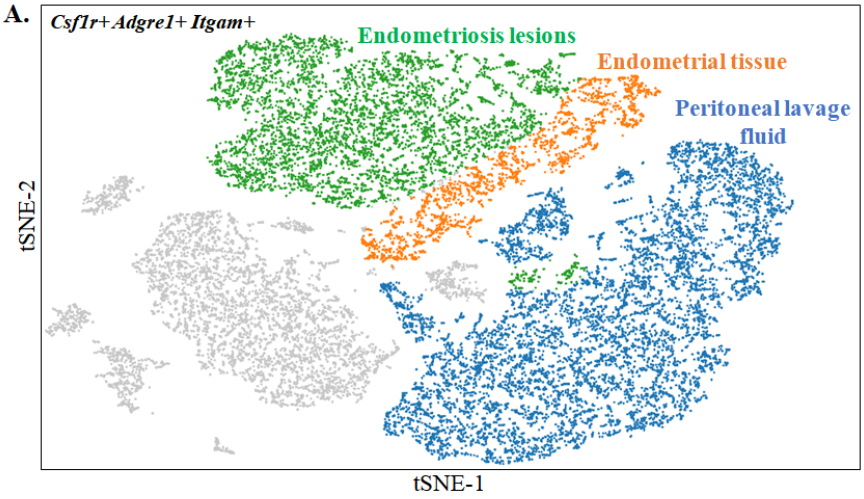


Figure 5.2. tSNE analysis of CD45+ cells from endometriosis lesions, decidualised endometrial tissue and peritoneal lavage fluid from sham and endometriosis mice. ***A:*** *The four samples clustered together highlighting cell types across the samples. CD45+ leukocytes were defined based on know genes. Csf1r+, Adgre1+ and Itgam+ macrophages Cd19+, Cd79a+ and Ly6d+ B cells, Gzmc+, Gzme+, Gzmf+, Nkg7+ and Thy1+ NK cells and Cd3g+, Cd3d+, Ccl5+, Il2rb+ T cells were present in the samples. Endometriosis lesion macrophages clustered with both the endometrial tissue macrophages (area highlighted with a 1) and the peritoneal macrophages (area highlighted with a 2). PF = peritoneal lavage fluid.*

To determine whether endometriosis lesion resident macrophages possessed a distinct transcriptional profile, I compared macrophages from endometriosis lesions, donor endometrial tissue (eutopic endometrial macrophages) and peritoneal macrophages. First I manually separated the *Csf1r+ Adgre1+ Itgam+* macrophages into the three groups based on sample type using the 'Lasso selection' function in Loupe Cell Browser (*figure 5.3A*). As the endometriosis lesion sample and endometrial tissue sample demonstrated significant overlap, macrophages, which were present in this overlapped region, were classified as endometrial tissue to avoid any ambiguity. Using Loupe cell browser I then interrogated locally defining genes to identify candidates unique to each sample.

I found that macrophages within the three tissues had significantly different transcriptional profiles. In *figure 5.3B* I list the 20 most up-regulated gene transcripts in the tissues and a full list of differentially expressed genes can be found in *appendix 2*.

A.



B.

Tissue	Top 20 up-regulated genes
Endometrial tissue	*Il1b, Ly6C2, Ccl4, Hspa1a, Ms4a4c, Gatm, Rgs1, Isg15, Cxcl2, Cd83, Jun, Stmn1, Plac8, Ms4a6b, Dnajb1, Mmp12, Clec4n, Fos, Plbd1, Hmgn2*
Peritoneal lavage fluid	*Cxcl13, Slpi, Alox15, Padi4, Prtn3, Icam2, Fcna, Prg4, Saa3, Lrg1, Cd5l, Fabp4, Fn1, Ltc4s, Hgsnat, Mcemp1, F10, Emilin2, Itga6, Klf2*
Endometriosis lesions	***Ccl8, Gas6, Mrc1, Spp1***, *Ms4a7, Marcks, Wwp1, Abca1, Cbr2, Maf, Cd63, Stab1, Fcrls, Clec10a, Clec4a2, Hspa1b, Sepp1, Clec12a, Cltc, Dab2*

Figure 5.3. Differentially expressed genes in Csf1r+ Adgre1+ Itgam+ macrophages in endometrial tissue, peritoneal lavage fluid and endometriosis lesions. ***A:*** *tSNE plot of macrophages in endometriosis lesions, endometrial tissue and peritoneal lavage fluid. In order to determine differential gene expression between macrophages in the endometrium, peritoneal lavage fluid and endometriosis lesions, the Csf1r+ Adgre1+ Itgam+ cells were clustered into sample type using the 'Lasso selection' tool in Loupe Cell Browser 3.0.1.* ***B:*** *shows the top 20 genes differentially expressed by macrophage populations in each sample. Highlighted in bold are genes most highly up-regulated by endometriosis lesion macrophages which are depicted graphically in* ***figure 5.10****.*

Gene ontology analysis (performed using http://geneontology.org/) was utilised to gain insight into the function of endometrial, peritoneal and endometriosis lesion resident macrophages in my data. Endometrial macrophages up-regulated genes involved in positive regulation of hematopoietic stem cell migration (>100 fold enrichment), tolerance induction to self antigen (60.92 fold enrichment), monocyte

extravasation (48.73), antigen processing and presentation of peptide antigen via MHC class II (45.69 fold enrichment) and tissue regeneration (14.62 fold enrichment). Peritoneal macrophages up-regulated genes involved in cellular extravasation (26.05 fold enrichment), leukocyte cell-cell adhesion (17.36 fold enrichment), myeloid leukocyte migration (16.03 fold enrichment) and chemokine-mediated signalling pathways (15.44 fold enrichment). Endometriosis lesion resident macrophages up-regulated genes involved in positive regulation of hematopoietic stem cell migration (<100 fold enrichment), monocyte extravasation (92.90 fold enrichment), wound healing involved in inflammatory response (77.42 fold enrichment), cell migration involved in vasculogenesis (77.42 fold enrichment), regulation of macrophage inflammatory protein 1 alpha production (51.61 fold enrichment), positive regulation of monocyte differentiation (51.61 fold enrichment), macrophage proliferation (44.24 fold enrichment) and positive regulation of the neuroinflammatory response (38.71 fold enrichment).

To gain further insight into the transcriptional diversity of eutopic endometrial macrophages, peritoneal macrophages and endometriosis lesion macrophages, I produced a heat map of log2 fold gene changes across the samples. Notably, eutopic endometrial macrophages and endometriosis lesion resident macrophages showed the highest degree of similarity in gene expression changes and peritoneal macrophages were distinct from both of these (*figure 5.4*).

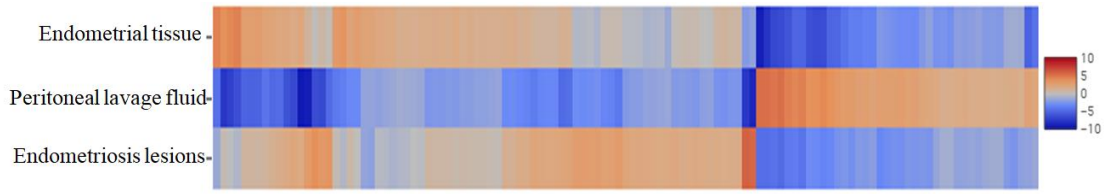


Figure 5.4. Heat map of Csf1r+ Itgam+ Adgre1+ macrophages in endometrial tissue, peritoneal lavage fluid and endometriosis lesions (Log2 fold changes)
Endometrial tissue macrophages and endometriosis lesion macrophages display similarities in gene expression patterns. Peritoneal lavage fluid macrophages are more genetically distinct from the endometrial and endometriosis lesion macrophages.

To determine the specificity of up-regulated genes in the endometriosis lesion sample to this tissue, the four most highly up-regulated genes were mapped to t-SNE plots of

all four samples (endometriosis lesions, endometrial tissue, peritoneal lavage fluid from sham and endometriosis mice) (*figure 5.5*). The tSNE plots largely demonstrated that expression of the *Ccl8*, *Gas6*, *Mrc1* and *Spp1* transcripts were highest in the endometriosis lesion sample. *Ccl8* was largely restricted to endometriosis lesion macrophages but was also expressed by lesion B, T and NK cells. *Gas6* expression was mostly restricted to endometriosis lesions however a population of endometrial macrophages also highly up-regulated this gene. *Mrc1* and *Spp1*expression was increased in endometriosis lesions and these genes were also expressed at lower levels in endometrial tissue and for the *Mrc1* transcript in the peritoneal lavage fluid sample also.

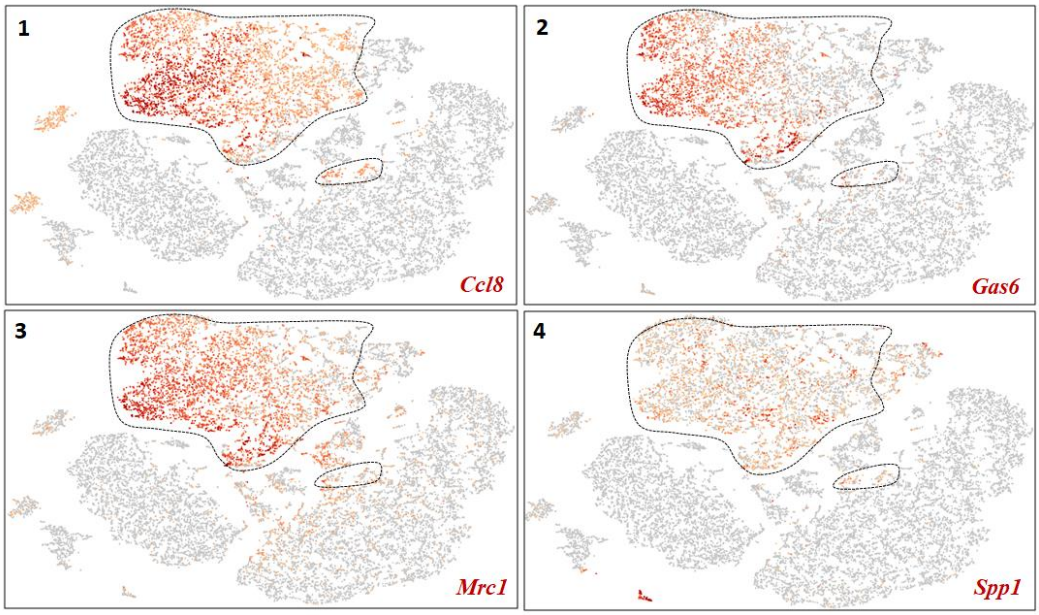


Figure 5.5. t-SNE plots of the four Most up-regulated genes in the endometriosis lesion macrophages across all of the single cell samples
Gene expression is shown where increased intensity in colour (red being most intense) indicates increased abundance of gene transcripts. Ccl8, Gas6, Mrc1 and Spp1 are significantly up-regulated in endometriosis lesion macrophages compared to eutopic endometrial and peritoneal macrophages.

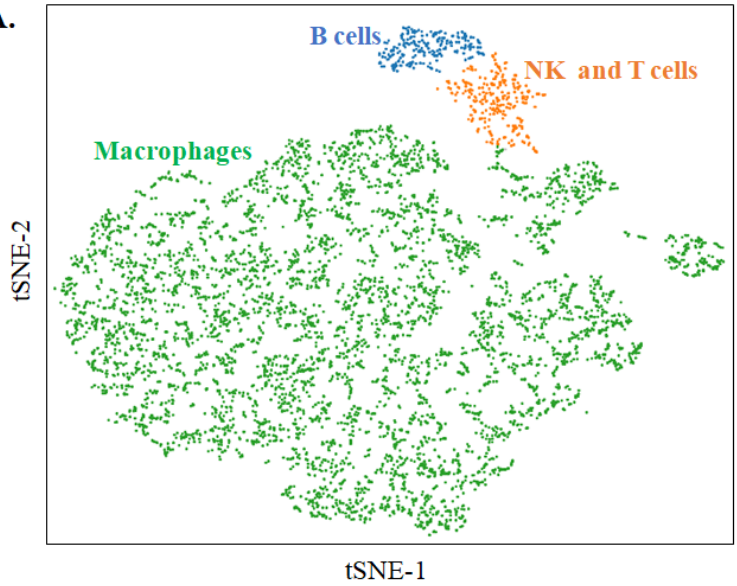
5.3.3 Endometriosis lesion resident macrophages exhibit transcriptional heterogeneity

In order to appreciate the true complexity of macrophage transcriptional diversity within endometriosis lesions, I visualized this sample as a single file within Loupe,

As tSNE plots are generated based on the relationship of each cell to one another, differential clusters are generated when each sample is visualised alone.

Initially, I visualised CD45+ leukocyte populations within the endometriosis lesions tissue. As expected, macrophages predominated in the tissue with smaller populations of B cells, NK and T cells also being present (*figure 5.6A*). The genes used to define CD45+ leukocyte clusters are listed in *figure 5.6B.*

A.



B.

Cell type	Defining Genes
B cells	*Cd79a, Cd79b, Ly6d, Ebf1, Mzb1*
T cells	*CD3g, CD3e, Cd3d, CCl5, Ptprcap, Il7r, Cd2*
NK cells	*NKg7, Thy1*
Macrophages	*Adgre1, Csf1*

Figure 5.6. Subsets of CD45+ leukocytes in mouse endometriosis lesions
Subsets of CD45+ leukocytes in the endometriosis lesions sample were defined using genes already known to be expressed by leukocyte populations in a similar manner as to figure 5.2. ***A:****tSNE plot of mouse endometriosis lesions sample showing clusters of CD45+ leukocytes. Macrophages, B cells, NK cells and T cells were present in the endometriosis lesion sample.* ***B:*** *Genes used to define CD45+ cell types within the endometriosis lesion sample.*

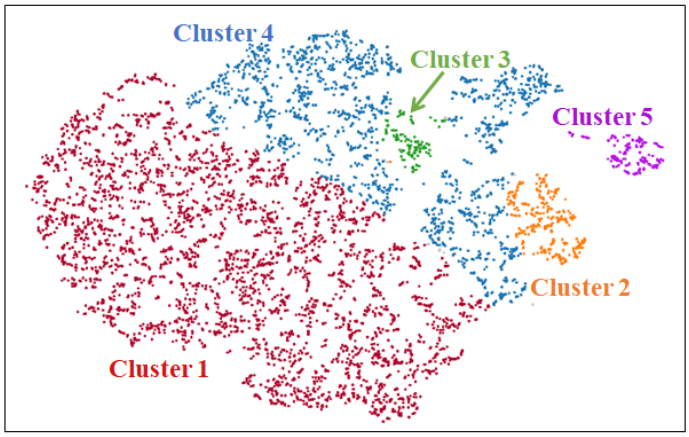
Adgre1+ Csf1r+ macrophages in the endometriosis lesions sample were subjected to graph based clustering using Loupe 3.0.1 software and clusters which exhibited restricted or increased gene expression of their most highly up-regulated genes compared to all other clusters were taken forward for analysis. In the lesion *Adgre1+*

Csf1r+ cells there were five main clusters of transcriptionally distinct macrophage populations (*figure 5.7A*). The top 20 up-regulated genes are listed in *figure 5.7B* and a full list of all the significantly differentially expressed genes can be found in *appendix 1*. The largest macrophage cluster, cluster 1, up-regulated a number of novel genes as well as genes such as *Igf1*, which we have recently published is important for macrophage-mediated neurogenesis in lesions (Forster*et al*., 2019). Notably, the most highly up-regulated gene in cluster 1, was *Gas6*. The increase in *Gas6* in lesion macrophages compared with eutopic endometrial and peritoneal macrophages identified in *figure 5.3* can thus be attributed to this sub-population of macrophages within the lesion, as well as up-regulation of the *Ccl8* transcript. Gene ontology analysis (performed using http://geneontology.org/) revealed cluster 1 up-regulated genes involved in positive regulation of steroid hormone biosynthetic process (63.52 fold enrichment), regulation of neuroinflammatory responses (34.30 fold enrichment), regulation of macrophage migration (20.92 fold enrichment) and tissue regeneration (17.50 fold enrichment).

The most highly up-regulated gene in cluster 2 was *Mmp12*, which is classically associated with tissue remodelling but has also been implicated in immune cell suppression (specifically T cells) in a cancer setting (Qu, Yan and Du, 2011). Gene Ontology analysis showed up-regulation of genes involved in positive regulation of chemokine (C-X-C motif) ligand 2 production (92.13 fold enrichment), neutrophil chemotaxis (40.54 fold enrichment), granulocyte chemotaxis (37.08), chemokine-mediated signalling pathways (28.15 fold enrichment), regulation of fibroblast proliferation (23.90 fold enrichment) and regulation of cytokine secretion (14.41 fold enrichment). Cluster 3 was the smallest macrophage cluster and up-regulated genes involved in positive regulation of cell-substrate adhesion (48.77 fold enrichment), cellular response to inorganic substances (27.84 fold enrichment), leukocyte migration (24.90 fold enrichment) and inflammatory response (13.89 fold enrichment). Cluster 4 up-regulated genes involved in cellular response to cytokine stimuli (11.27 fold enrichment). The most highly up-regulated gene, *Retnla*, codes for the cytokine RELMα which has been identified as a pro-inflammatory factor in a mouse model of colitis, where RELMα stimulated IL-6 and TNF-alpha release and inhibited release of the anti-inflammatory cytokine IL-10, perpetuating

pathology (Munitz *et al.*, 2008). Cluster 5 is defined by genes associated with proliferation, DNA replication and preventing apoptosis, for example *2810417H13Rik*, *Top2a*, *Birc5*, and *Stmn1*, the most highly up-regulated genes. Gene ontology analysis in this cluster identified that up-regulated genes are involved in spindle organisation (42.88 fold enrichment), nuclear division (20.42 fold enrichment), cell cycle (14.44 fold enrichment), mitotic cell cycle process (19.35 fold enrichment), cell division (14.52 fold enrichment) and chromosome organisation (12.20 fold enrichment).

A.



B.

Cluster	Top 20 up-regulated genes
1	***Gas6, Ccl8, Igfbp4, Cbr2***, *Wwp1, Stab1, Folr2, Maf, Sepp1, Mrc1, F13a1, Dab2, Clec10a, Fcgrt, Serinc3, Igf1, Fcrls, Abca1, Pf4, Ly6e*
2	***Mmp12, Alas1, Cxcl3, Prdx1***, *Lpl, Esd, Cd9, Cd36, Gpnmb, Cstb, Spp1, Lgals3, Clec4d, Sod2, Fabp5, Capg, Pgam1, Fn1, Anxa1, Itgb2*
3	***Alox15, Prg4, Cxcl13, Saa3***, *Prtn3, Slpi, Icam2, Padi4, Lrg1, Mcemp1, Cd5l, Thbs1, Fabp4, Fn1, Itga6, Emilin2, Ltc4s, Smpdl3a, Ednrb, Klf2*
4	***Retnla, Lsp1, Tmsb10, Id2***, *S100a11, Fabp4, Cd52, Cxcl2, Lyz1, S100a4, Clec4b1, S100a6, Mcemp1, Gm8730, Crip1, Gm2a, Srgn, Nfkbia, Fn1, Rplp0*
5	***2810417H13Rik, Top2a, Birc5, Stmn1***, *Tmpo, Tuba1b, Tubb5, Pcna, Ube2s, Slpbp, H2afv, H2afz, Anp32bm, Nucks1, Dek, Ptma, Ranbp1, Nap1l1, Ran, Srsf3*

Figure 5.7. Subsets of Csf1r+ Adgre1+ macrophages in mouse endometriosis lesions

A:*tSNE plot of Adgre1+ Csf1r+ macrophages in mouse endometriosis lesions showing clustering of 5 transcriptomically distinct sub-populations.* ***B:*** *Table of the top 20 up-regulated genes in each cluster of Adgre+ Csf1r+ macrophages in mouse endometriosis lesions. The four most highly up-regulated genes are highlighted in bold and displayed graphically in figure 5.5.*

In order to demonstrate that the up-regulated genes in each macrophage cluster were cluster specific, the four most up-regulated genes in each population were mapped

onto tSNE plots to determine gene expression over the endometriosis lesion sample (*figure 5.8*). Up-regulated genes were either up-regulated or expressed almost exclusively (in the cases of clusters 3 and 5) to the relevant cluster, supporting that the clusters produced represent 'real' transcriptionally distinct populations.

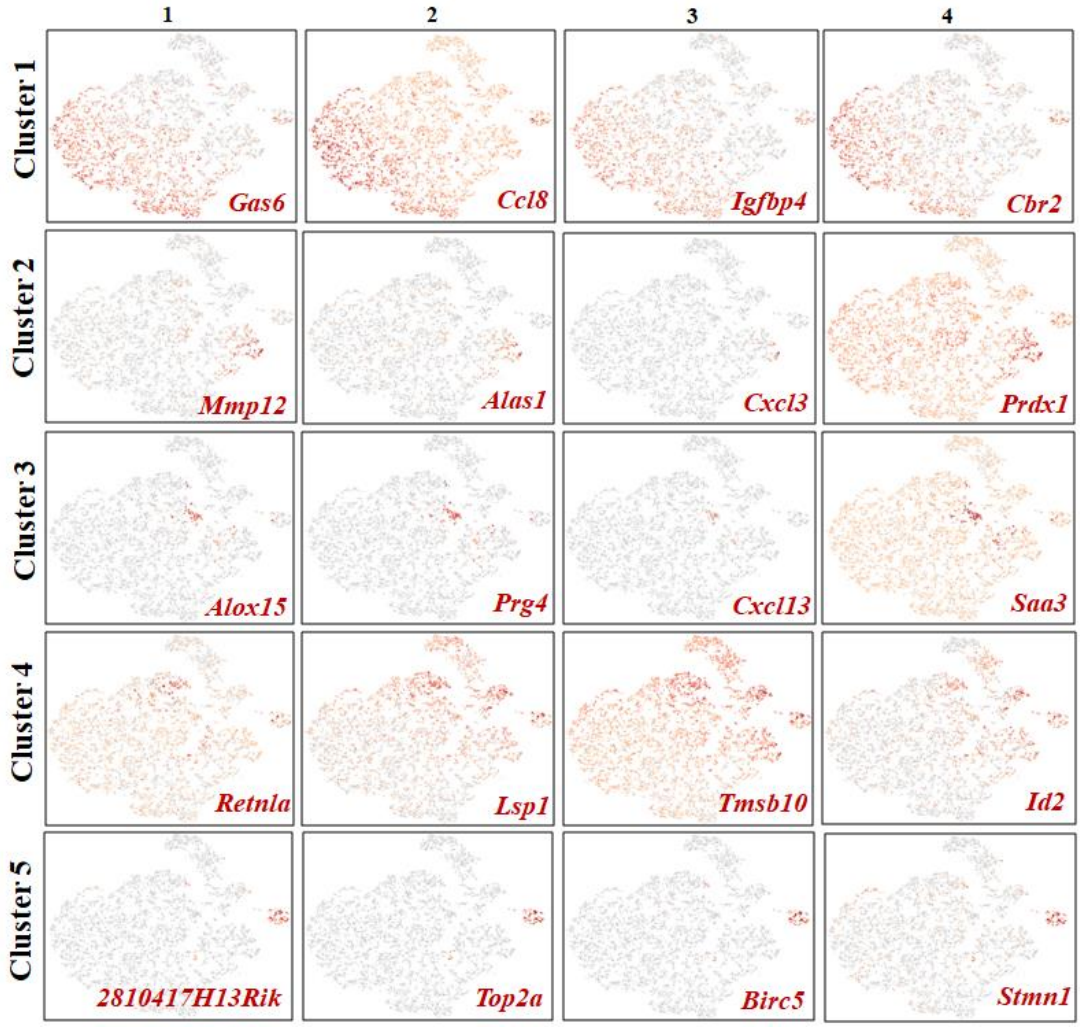


Figure 5.8. t-SNE plots for most highly up-regulated genes in clusters of endometriosis lesion macrophages

tSNE plots of the most highly up-regulated genes which define macrophage clusters 1-5. Gene expression is shown where increased intensity in colour (red being most intense) indicates increased abundance of gene transcripts. Selected genes demonstrate increased gene expression in the relevant macrophage cluster or gene expression is largely restricted to the cluster.

To further analyse the transcriptional diversity between lesion resident macrophage clusters, a heat map showing log2 fold changes in transcript expression was produced for clusters 1-5 (*figure 5.9*). Heat map analysis demonstrated differential expression

patterns between the clusters whereby clusters 2 and 4 demonstrated the highest degree of similarity.

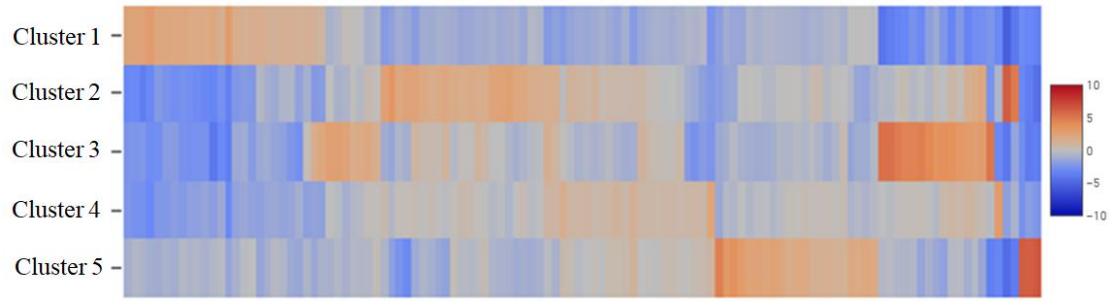


Figure 5.9. Heat map of differentially expressed genes in clusters 1-5 of endometriosis lesion resident macrophages (Log2 fold changes)
Endometriosis lesion resident macrophages exhibited transcriptional heterogeneity which is reflected in the differential genetic profiles presented in this heat map.

5.3.4 Endometriosis lesion macrophages express the Gas6 receptor Axl suggesting the potential for paracrine signalling

Gas6 is the most highly up-regulated transcript in endometriosis lesion macrophage cluster 1, which is the largest macrophage cluster within the lesion tissue (refer to *figure 5.4* for tSNE clustering). When lesion macrophages were compared with eutopic endometrial and peritoneal macrophages, *Gas6* was the second most highly up-regulated gene in endometriosis lesions (*figure 5.3*).The *Gas6* gene encodes for the Gas6 protein which binds with a high affinity to the Axl receptor, encoded by the *Axl* gene (Korshunov, 2012). Due to the fact that endometriosis lesion resident macrophages highly up-regulate the *Gas6* transcript, I wanted to ascertain whether lesion macrophages also expressed the Gas6 receptor, *Axl*, in order to infer as to whether paracrine signalling could be occurring within the lesion microenvironment. tSNE plots mapping the expression of both *Gas6* and *Axl* were produced in the endometriosis lesions sample which demonstrated high expression of both *Gas6* and *Axl* in lesion macrophage populations (*figure 5.10A/B*). *Gas6*was largely expressed by cluster 1 as previously demonstrated in *figure 5.5*. Interestingly, *Axl* was highly expressed by lesion macrophages, residing in the other clusters, although *Axl* was also highly up-regulated in some cluster 1 cells suggesting the autocrine signalling might take place as well.

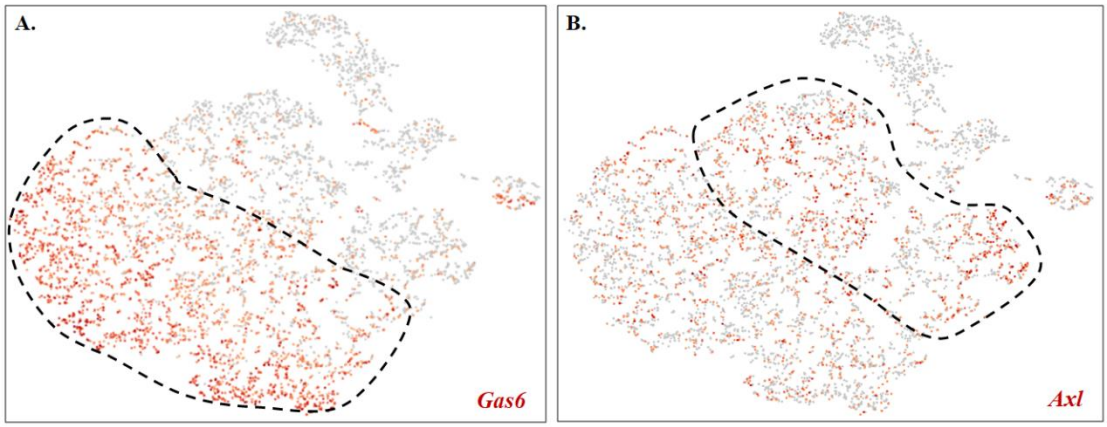


Figure 5.10. Endometriosis lesion macrophages express the Gas6 ligand and the Gas6 receptor Axl*. Gene expression is shown where increased intensity in colour (red being most intense) indicates increased abundance of gene transcripts.* ***A:****tSNE plot of Gas6 expression across the endometriosis lesion sample. The Gas6 transcript is highly expressed in a large proportion of lesion macrophages (circled).* ***B:****tSNE plot of Axl expression across the endometriosis lesion sample. The Gas6 receptor Axl is highly expressed by lesion macrophages (circled).*

5.3.5A A population of lesion macrophages express the proliferation marker *Mki67*

Ki67 is expressed by dividing cells and thus is commonly utilised as a marker of proliferation (Juríková *et al.*, 2016). To investigate whether endometriosis lesion macrophages have the ability to proliferate in our model, I mapped the *Mki67*gene which codes for Ki67 onto a tSNE plot of the endometriosis lesions sample. *Mki67* was almost exclusively expressed by macrophage cluster 5 (circled), identifying that this population represents a sub-population of macrophages within endometriosis lesions which are able to proliferate *in situ* (*figure 5.11*). Cluster 5 is also defined by up-regulation of a number of replication specific genes, such as *2810417H13Rik* (DNA replication and repair), *Top2a* (important for DNA replication), *Birc5* (inhibits apoptosis) and *Stmn1* (cell cycle regulation). Identification of ki67 at the protein level in macrophages in endometriosis lesions in our mouse model was attempted using immunohistochemistry (co-staining of ki67 and F4/80). This would provide additional evidence that lesional macrophages are proliferative. However, this staining was not successfully optimised within the time frame of my PhD (data not shown).

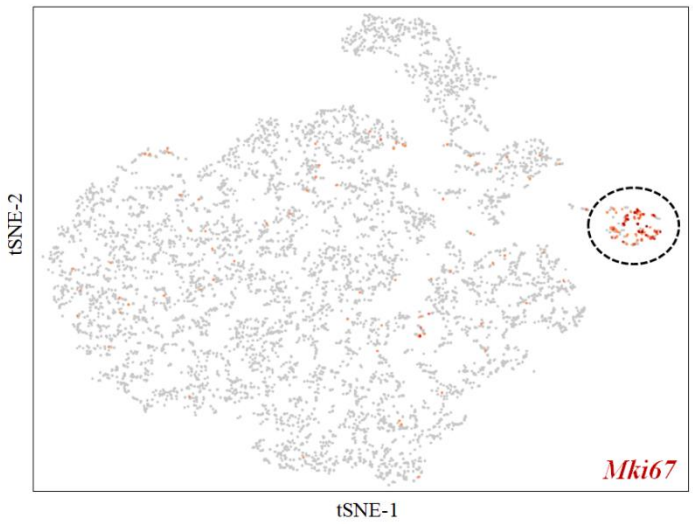


Figure 5.11. Cluster 5 is characterised by expression of the proliferation marker Mki67

tSNE plot of mouse endometriosis lesions showing expression of the Mki67 gene with cluster 5 circled. Gene expression is shown where increased intensity in colour (red being most intense) indicates increased abundance of gene transcripts. Mki67 expression was almost exclusively restricted to cluster 5.

5.4 Summary

In this chapter I have demonstrated that endometriosis lesion resident macrophages can be classified into sub-populations based on cell surface marker expression. Furthermore, I have used single cell RNA sequencing to show that endometriosis lesion resident macrophages are transcriptionally diverse from eutopic endometrial and peritoneal macrophages. I interrogated macrophage transcriptional heterogeneity within endometriosis lesions and characterised five major populations of lesion macrophages, demonstrating that lesion macrophages are a heterogeneous population.

Using Loupe 3.0.1 software I observed that 5 key populations of macrophages are present in endometriosis lesions in our mouse model. I discerned that a cluster of lesion macrophages (cluster 5) up-regulated the proliferation marker *Mki67* along with a number of proliferation specific genes (*2810417H13Rik, Top2a, Birc5, Stmn1*), suggesting that this population exhibits *in situ* proliferation. Gene ontology analysis of cluster 5 identified genes involved in spindle organisation, nuclear division, the cell cycle, mitotic cell cycle process, cell division and chromosome

organisation, further supporting this. One of the most highly up-regulated genes by the lesion macrophages was *Gas6*, and lesion macrophages also up-regulated the *Axl* gene which codes for the high affinity *Gas6* receptor, Axl. This data suggests the potential for paracrine signalling between macrophage populations within endometriosis lesions.

Overall, in this chapter I have characterised that endometriosis lesion macrophages exhibit a higher level of complexity than previously appreciated and defined up-regulate genes that may be implicated in disease development. This data provides an insight into macrophage heterogeneity, signalling and proliferative renewal within lesions.

5.5 Discussion

The data presented in *figure 5.1* demonstrated that macrophages in endometriosis lesions can be stratified into sub-populations using analysis of markers used in flow cytometry which have typically been used to represent 'extremes' of macrophage activation, either as 'anti-inflammatory' or 'pro-inflammatory' markers (Martinez and Gordon, 2014). Eutopic endometrial macrophages could be separated into eight populations using the markers CCR2, MHC II and CD86, however in endometriosis lesions, endometrial-derived macrophages formed only one major population. This suggests that heterogeneity in this macrophage population is lost upon incorporation into endometriosis lesions. It is possible that only one macrophage population is persisting in lesions, or that macrophage phenotype is modulated upon incorporation into the novel microenvironment of the endometriosis lesion. We know that in cancer, macrophage phenotype is modulated upon incorporation into the novel microenvironment of the tumour, where growth factors and cytokines modulate macrophages to a 'tumour specific' phenotype (Quatromoni and Eruslanov, 2012; Wu and Zheng, 2012; Cassetta *et al.*, 2019). The fact that a large proportion of recipient derived, infiltrating macrophage populations also shared the same phenotype as the endometrial-derived macrophages suggests re-programming of macrophage phenotype within the endometriosis lesion microenvironment. This is depicted schematically in *figure 5.12*. A key limitation of these studies is the difficulty to infer as to whether the macrophage populations characterised reflects the true

heterogeneity or phenotypes of macrophages, due to the use of only four surface markers. I addressed this limitation by employing single cell RNA sequencing.

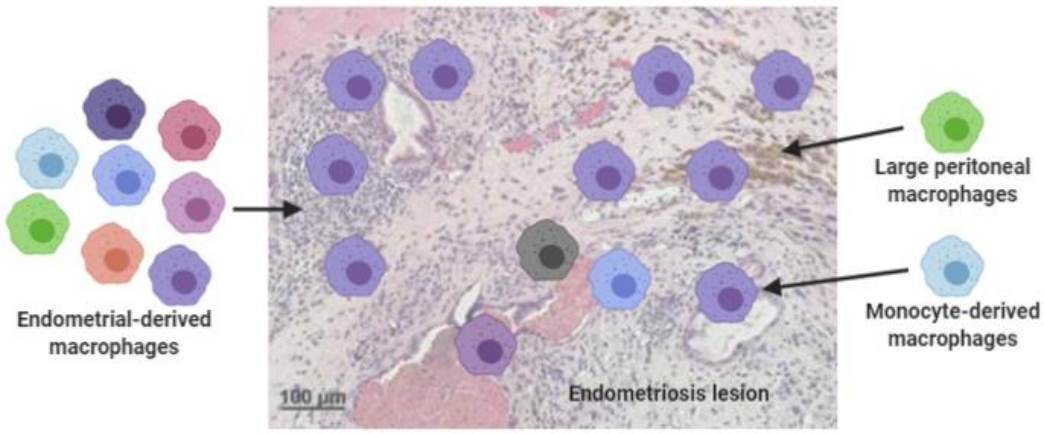


Figure 5.12. Schematic Summarising Macrophage Sub-populations in Eutopic Endometrial Macrophages and Endometrial-derived and Recruited Macrophage Populations in Endometriosis Lesions
This schematic summarises data from figure 5.1. At flow cytometric analysis, eutopic endometrial macrophages in the decidualised endometrium formed 8 sub-populations based on the CCR2, MHC II and CD86 markers. Incorporation into endometriosis lesions lead to a reduction in the heterogeneity of this population. Recruited macrophage populations formed four sub-populations using the CCR2, MHC II and CD86 markers.

I analysed macrophage populations from the decidualised eutopic endometrium, endometriosis lesions and peritoneal lavage fluid by single cell RNA sequencing. Transcriptional analysis showed that endometriosis lesion resident macrophages exhibit differential gene expression compared to eutopic endometrial and peritoneal macrophages, however they shared the highest degree of similarity to the eutopic endometrial macrophages. Gene ontology testing identified shared functions between endometrial and endometriosis lesion macrophages such as positive regulation of haematopoietic stem cell migration and wound healing/tissue regeneration responses. This analysis highlights that functionally, endometrial and lesion resident macrophages may be more similar, compared with peritoneal macrophages which up-regulated genes involved in processes such as cellular extravasation and leukocyte cell-cell adhesion. Functions such as wound healing and recruitment of haematopoietic cells may be important to the pro-disease role of macrophages in stimulating the growth and development of endometriosis lesions (Bacci *et al.*, 2009; Greaves *et al.*, 2015). The similarity between endometrial and lesion resident

macrophages is perhaps unsurprising as endometriosis lesions are derived from decidualised endometrial tissue in our mouse model (endometrial tissue is injected into the peritoneal cavity where it adheres to form lesions). Macrophages modulate their phenotype and gene expression based on the signals they encounter in their local micro-environment (Wynn, Chawla and Pollard, 2013). Thus, macrophages present within endometriosis lesions may encounter a more 'similar' microenvironment to eutopic endometrial macrophages compared with macrophages placed in the peritoneal cavity. The placement of endometriosis lesions in the peritoneal cavity in our model, most commonly on the peritoneal wall, exposes lesions to cytokines, chemokines and growth factors in peritoneal fluid, which likely modulate the macrophage transcriptome.

To date there have been no transcriptomic studies on endometriosis lesion macrophages and thus this sequencing data provides a first insight into the transcriptional profile of these cells. The most highly up-regulated genes in the lesion macrophage population are *Ccl8*, a monocyte/macrophage chemokine (Asano *et al.*, 2015), *Gas6*, a mitogen (Loges *et al.*, 2010), *Mrc1*, typically a marker of anti-inflammatory macrophages (Martinez and Gordon, 2014), and *Spp1*, which is implicated in macrophage recruitment and modulating macrophage cytokine production (Castello *et al.*, 2017). Important parallels can be drawn from the transcriptomic profile of endometriosis lesion macrophages and tumour associated macrophages, which have been shown to play similar roles such as angiogenesis and stimulating stromal cell proliferation and survival in a disease setting (Bacci *et al.*, 2009; Noy and Pollard, 2014; Shao *et al.*, 2016). One key parallel is the up-regulation of the mitogen *Gas6.*

In this chapter I demonstrated that lesion macrophages up-regulated *Gas6* and this was the most highly up-regulated gene in macrophage cluster 1, the largest macrophage cluster in lesion tissue. Lesion macrophages up-regulated both *Gas6* and the high affinity Gas6 receptor *Axl*. Interestingly, expression of the Gas6 receptor *Tyro3* was almost absent from endometriosis lesions and the Mer receptor (*Mertk*) was expressed at a low level in a small number of cells (data not shown). These receptors have a reduced affinity for the Gas6 ligand compared with the Axl receptor

(Axl>Tyro3>Mer) (Lemke and Rothlin, 2008). Gas6/Axl signalling has been investigated in a number of disease settings. For example, co-expression of high levels of Gas6 and Axl in glioma tumours is associated with reduced survival (Hutterer *et al.*, 2008) and the Gas6 ligand is able to increase the proliferation and survival of cancer cell lines *in vitro* (Van Ginkel *et al.*, 2004; Sainaghi *et al.*, 2005). An elegant study by Loges *et al* revealed insight into the role of macrophage-derived Gas6 in cancer. Using Gas6 knockout mice they demonstrated, in models of breast and pancreatic cancer, that infiltrating macrophages were educated by the tumour microenvironment to produce increased levels of Gas6, which in turn increased the proliferation and survival of tumour cells. They also noted that in five different cancer cell lines Axl was more highly expressed than the Tyro3 or Mer receptors (Loges *et al.*, 2010). In my data, *Gas6* was highly up-regulated in endometriosis lesion macrophages compared with eutopic endometrial and peritoneal macrophages. This suggests that this gene is specifically up-regulated within the endometriosis lesion micro-environment, however the mechanism by which this gene is up-regulated or the role that Gas6/Axl signalling may play in endometriosis is entirely unknown.

I investigated the heterogeneity of endometriosis lesion macrophages and demonstrated that five sub-populations were present within the tissue, which exhibited differential transcriptomic profiles. Previous investigations into macrophage phenotype within endometriosis lesions have largely relied on immunohistochemical techniques which do not appreciate the possibility for macrophage heterogeneity (Bacci *et al.*, 2009; Johan *et al.*, 2019). Notably, these studies have also largely relied on the M1/M2 paradigm, however in my data lesion macrophage populations up-regulated a number of both 'M1' and 'M2' markers, supporting the notion that this is a vastly over-simplified classification of macrophage phenotype (Martinez and Gordon, 2014). In tumours, multiple populations of macrophages exist (summarised in *figure 5.13*) which promote differential aspects of disease development (Qian and Pollard, 2010). In this way, macrophage populations are 'specialised' to promote specific aspects of disease development. It is possible, therefore, that transcriptionally divergent macrophage populations within endometriosis lesions fulfil differential roles during disease

development and within the lesion microenvironment. From the data presented in this chapter, it is possible to infer the roles that these macrophage populations may fulfil.

Gene ontology analysis demonstrated that cluster 1 up-regulated genes involved in positive regulation of steroid hormone biosynthetic processes, regulation of neuroinflammatory responses, regulation of macrophage migration and tissue regeneration. Data from previous studies has already demonstrated that a number of these functions are central to endometriosis pathophysiology. For example, we know that endometriosis lesions locally produce estrogen, which stimulates the growth of lesions (Noble *et al.*, 1997; Zeitoun *et al.*, 1998; Attar and Bulun, 2006). We also know that macrophages are central to neurogenesis in lesions, which contributes to pain symptoms in mice and is mediated by *Igf1*, also up-regulated by this cluster (Greaves *et al.*, 2015; Forster *et al*., 2019). Depletion of peritoneal macrophages using clodronate liposomes reduces macrophage recruitment to lesions and subsequently fewer endometriosis lesions are formed, highlighting the importance of macrophage recruitment from the peritoneal cavity in the development of lesions (Bacci *et al.*, 2009; Forster *et al.*, 2019). In a disease setting, tissue regeneration is commonly a 'pro-disease' function which can maintain the health of pathological tissue rather than eliciting an immune response to clear it (Wynn, Chawla and Pollard, 2013). A number of genes up-regulated by this cluster, including *Gas6* and *Ccl8*, have also been implicated in supporting the growth and survival/metastasis of cancer cells (Loges *et al.*, 2010; Cassetta *et al.*, 2019). These ligands could therefore be important in supporting the survival and dissemination of endometrial stromal cells within the peritoneal cavity. Thus, it is clear that this lesion macrophage cluster up-regulates a number of genes which are likely to contribute to pathology, and the fact that cluster 1 is the largest population of lesion macrophages further emphasises the importance of this macrophage population within the lesion microenvironment.

Cluster 2 represents a smaller lesion macrophage population which, at gene ontology analysis, up-regulated genes involved in positive regulation of chemokine (C-X-C motif) ligand 2 production, neutrophil chemotaxis, granulocyte chemotaxis, chemokine-mediated signalling pathways, regulation of fibroblast proliferation and

regulation of cytokine secretion. Zhang *et al* noted that CXCL2 was up-regulated in the peritoneal fluid of mice with endometriosis-like lesions (heterologous model), whereby CXCL2 was important for the recruitment of myeloid-derived suppressor cells to the peritoneal cavity (Zhang *et al.*, 2018). CXCL2 is also known to mediate the recruitment of neutrophils during tissue inflammation (De Filippo *et al.*, 2013), which is synergistic to the up-regulation of genes involved in neutrophil chemotaxis at gene ontology analysis. Takamura *et al* reported that neutrophils are central to the growth of endometriosis lesions in a heterologous mouse model, but use of an anti-Gr1 antibody did not account for the expression of Gr1 on mouse monocytes (Takamura *et al.*, 2016). The role of neutrophils in endometriosis thus remains largely elusive, although my data suggests that neutrophil chemotaxis is, at least in part, mediated by a population of lesion resident macrophages. Interestingly, cluster 2 also up-regulated genes involved in regulation of fibroblast proliferation. Fibroblasts are known to play roles in regulating the extracellular matrix and wound healing, and a number of diseases are associated with dysregulation in fibroblast function and an altered wound healing responses (McAnulty, 2007). Fibroblasts in the eutopic endometrium of women with endometriosis exhibit progesterone resistance, inhibited decidualisation and a pro-inflammatory phenotype compared to healthy controls, suggesting that fibroblast dysregulation is a hallmark of endometriosis (Barragan *et al.*, 2016). My data therefore suggests that a macrophage-fibroblast cross talk may occurr within endometriosis lesions, and this could have the potential to exacerbate disease by regulating the extracellular matrix and wound healing functions of fibroblasts to perpetuate survival and health of endometriosis lesions, although functional studies investigating this potential cross talk are required. Furthermore, the most highly up-regulated gene in cluster 2 is *Mmp12* (matrix metallopeptidase 12; Mmp12). Mmp12 is involved in breakdown of the extracellular matrix, further supporting the hypothesis that this macrophage population may be implicated in tissue remodelling in lesions.

Cluster 3 is the smallest population of macrophages in endometriosis lesions in our mouse model, and at ontology analysis up-regulated genes involved in positive regulation of cell-substrate adhesion, cellular response to inorganic substances, leukocyte migration and inflammatory response. A number of genes up-regulated by

this cluster relate to modulation of the inflammatory response. The most highly up-regulated gene in this population is *Alox15*, which is an enzyme involved in resolution of inflammation (Tian *et al.*, 2017). In macrophages, *Alox15* has been shown to be induced through interaction with apoptotic cells (Stables *et al.*, 2011). This suggests that this macrophage population may be defined apoptotic activity. *Alox15* is also thought to control the uptake of apoptotic cells by macrophages, limiting apoptotic reponses and thus preventing inappropriate immune responses (Uderhardt *et al.*, 2012). It is possible that this gene could act to suppress apoptotic clearance of ectopic endomtrial cells by macrophages, but functional studies are required to investigate this. This hypothesis is supported by the fact that peritoneal macrophages in women with endometriosis have reduced phagocytic capacity (Wu *et al.*, 2005). *Saa3*, the fourth most highly up-regulated gene in this cluster, is a gene involved in response to inflammation by recruitment of immune cells and regulation of the extracellular matrix, and secretion of the Saa3 protein has been shown to be important for tumour growth in both pancreatic and breast cancer (Djurec *et al.*, 2018; Ignacio *et al.*, 2019). *Cxcl13*, the third most highly up-regulated gene, is involved in the chemotaxis of B cells, and has been implicated in the recruitment of B cells to tumours and subsequent stimulation of tumour development (Tan *et al.*, 2018), suggesting *Cxcl13* could play a similar role in endometriosis lesions. Overall, taking into account gene ontology analysis and genes up-regulated by cluster 3, this macrophage population appears play roles in controlling the inflammatory response within lesions as well as mediating the recruitment of other leukocytes (specifically B cells), to lesions. Control of inflammation may be an important 'pro-disease' function within lesions, as escape of immune clearance in the peritoneal cavity is a key hallmark of endometriosis (Burney and Giudice, 2012).

Furthermore, through gene ontology analysis cluster 4 was shown to up-regulate genes involved in cellular responses to cytokine stimuli. The most highly up-regulated gene, *Retnla*, is implicated as a pro-inflammatory cytokine in a mouse model of colitis which stimualted development of pathology (Munitz *et al.*, 2008), although *Retnla* has traditionally been defined as a 'M2' macrophage cytokine (Nair *et al.*, 2009). The role this cytokine may play in endometriosis is currently unknown. Notably, cluster 4 up-regulated a number of genes in the S100 family of calcium-

binding proteins, such as *S100a11*, *S100a4* and *S100a6*. Dysregulated expression of the *S100* genes is assocaited with many cancers (Bresnick, Weber and Zimmer, 2015). In a number of cancer settings, up-regulation of *S100* genes has been shown to stimulate stromal cell proliferation, angiogenesis, metastasis and immune evasion in the tumour microenvironment (Bresnick, Weber and Zimmer, 2015). In endometriosis, S100A4 and S100p have been shown to be up-regulated in ectopic endometrial deposits from women, and this was proposed to enhance invasiveness and establishment of lesions (Hapangama *et al*., 2011). These proteins could therefore be involved in endometriosis pathophysiology, and further research of their role in the aetiology of disease could be of clinical interest. Furthermore, *Lsp1*, the second most highly up-regulated gene has been shown, in rheumatoid arthritis, to be a negative regulator of T cell chemotaxis (Hwang *et al.*, 2015). We know that in tumours, macrophages prevent the chemotaxis of T cells to the tumour in order to perpetuate the growth and development of tumours (Peranzoni *et al.*, 2018), thus, it is possible that macrophage-T cell cross-talk is also implicated within endometriosis lesions to encourage the growth and development of lesions. Overall, cluster 4 up-regulates a number of genes which have the potentail to stimulate the growth and development of endometriosis lesions, however functional studies are crucially required to discern if these genetic aberrations do indeed contribute to the pathophysiology of endometriosis.

Importantly, in this chapter I established that a sub-population (cluster 5) of endometriosis lesion resident macrophages highly up-regulated the proliferation marker *Mki67* along with a number of genes which are involved in cellular proliferation. Gene ontology analysis in this cluster revealed up-regulation of genes involved in many proliferation specific functions such as nuclear division, cell cycle associated genes and cell division. In chapter 3 I demonstrated that the lesion macrophage population is derived from endometrial tissue macrophages and infiltrating large peritoneal macrophages and Ly6C+ monocytes. I have now characterised that lesion macrophages are also maintained by *in situ* proliferation. In a mouse model of pancreatic ductal adenocarcinoma, tumour macrophages were seeded by circulating Ly6C^{hi} monocytes but also maintained by tissue resident macrophages which expanded by *in situ* proliferation (Zhu *et al.*, 2017). In my data

the origin of the proliferative cell population is un-characterised, and thus could incorporate any of the macrophage populations present within the lesion tissue. Large peritoneal macrophages are able to proliferate in the peritoneal cavity (Cassado, D'Império Lima and Bortoluci, 2015), however whether they maintain this capacity within endometriosis lesions is speculative. The proliferative capacity of macrophages in the endometrium is currently poorly understood. Further studies will therefore be important in understanding the dynamics of macrophage *in situ* proliferation within endometriosis lesions and how this population relates to disease development.

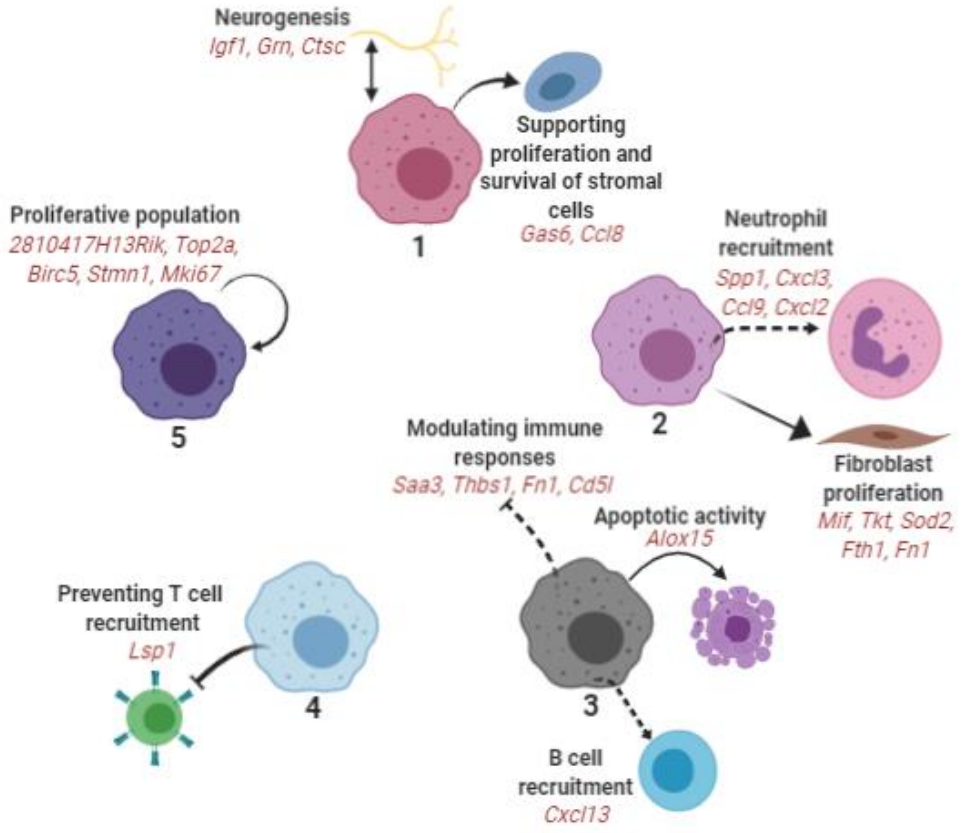


Figure 5.13. Endometriosis lesion resident macrophages are phenotypically heterogeneous and may fulfill distinct roles within the endometriosis lesion microenvironment. *I demonstrated, using single cell RNA sequencing, that endometriosis lesion resident macrophages in our mouse model were phenotypically heterogeneous, forming 5 transcriptionally diverse macrophage populations (labelled 1-5 in figure, referring to cluster number). Using gene ontology analysis and data from studies in other disease states, I was able to hypothesise the roles these macrophage populations may fulfill within endometriosis lesions. Note that these are hypothesised and not proven roles. The possible role that these macrophages may play within endometriosis lesions is in black writing and the genes associated with this function are shown in red below.*

Overall, in this chapter I demonstrate that, in our mouse model, endometriosis lesion resident macrophages are a phenotypically heterogeneous population with distinct transcriptional profiles. I also begin to infer as to the roles these differential macrophage populations may play in endometriosis pathophysiology, however further studies are required to (1) decipher if the populations described represent distinct populations at the protein level and (2) to characterise the roles these macrophage populations play in disease development using functional studies.

Chapter 6 - Discussion

6.1.1 Peritoneal monocyte and macrophage populations in endometriosis

In chapter 3 I characterised peritoneal monocyte/macrophage populations in our mouse model and demonstrated that whilst large and small peritoneal macrophages remain unperturbed, Ly6C^{hi} monocytes are increased in the peritoneal cavity at 2 weekspost tissue injection. The increase in Ly6C^{hi} monocytes in endometriosis thus illustrates the ability for endometriosis lesions to elicit extravasation of this cell population into the cavity. These results appear contradictory as inflammation in the peritoneal cavity is commonly associated with both an increase in Ly6C^{hi} monocytes and small peritoneal macrophages, which are pro-inflammatory in nature (Ghosn *et al.*, 2010), and endometriosis has been classically defined as an inflammatory disease (Burney and Giudice, 2012). In mouse models of peritonitis, we know that the macrophage disappearance reaction occurs in a predictable manner (Cassado *et al.*, 2011), however this does not appear to be the case in our mouse model of endometriosis. In these models, irritants such as zymosan are used to induce an inflammatory response in the peritoneal cavity (Cassado *et al.*, 2011; Bain and Jenkins, 2018). In a mouse model of endometriosis which inoculated naive, non-decidualised tissue into the peritoneal cavity, Yuan *et al* reported a decrease in LPM and increase in SPM in the peritoneal cavity of mice with endometriosis (Yuan *et al.*, 2017b). Whilst we injected decidualised endometrial tissue into the peritoneal cavity of mice in our mouse model, we did not see a similar inflammatory response; the key difference between these studies being the use of decidualised or non-decidualised endometrial tissue. This suggests that factors within the decidualised endometrial tissue are able to modulate the response of the immune system to its presence in the peritoneal cavity, and this is unique to endometrial tissue which has undergone differentiation into 'menses-like' tissue. Another factor which must be taken into consideration in our mouse model is the use of estradiol valerate supplementation in recipient mice, leading to superphysiological levels of estrogen. Estrogen has been shown to increase the proliferative capacity of macrophages and modulate their phenotype to be more 'wound-healing' like (Pepe *et al.*, 2017). Thus, in our mouse model, the number and phenotype of peritoneal macrophages may be modulated such that the macrophage disappearance reaction may not occur in a predictable manner, as with other models where estrogen was not supplemented (Cassado *et al.*, 2011;

Yuan *et al.*, 2017b). I observed a significant increase in the LPM population in sham mice at a 1 week time point, suggesting that superphysiological levels of estrogen do indeed cause proliferation of this tissue resident population. A modest but not significant increase in the LPM population of mice with endometriosis was also observed at this time point. Thus, the absence of the macrophage disappearance reaction in our mouse model of endometriosis is likely attributed to the use of decidualised endometrial tissue, but also to superphysiological levels of estrogen.

Overall, my results suggest that the presence of ectopic endometrial tissue (lesions) could cause the accumulation of specific subsets of immune cells (i.e. Ly6C^{hi} monocytes), rather than eliciting that body's 'normal' response to an inflammatory stimulus. This differential immune response may potentially allow lesions to evade immune clearance whilst also harnessing the immune system to stimulate disease. The role that Ly6C^{hi} monocytes are playing in disease however is currently unknown, and the mechanisms by which decidualised endometrial tissue may be able to modulate the immune response in the peritoneal cavity remains elusive.

6.1.2 Lesion resident macrophages comprise endometrial-derived and recruited populations

In chapter 3 I characterised the origins of lesion resident macrophages. Using fate maping studies, I demonstrated that 16% of lesion macrophages are derived from the endometrium and 84% are recruited to the tissue. In diseases such as cancer, macrophages within different ontogenies have been shown to play distinct roles in disease development (Zhu *et al.*, 2017), therefore it is possible that macrophages of different origins are playing diverse roles in the development of endometriosis lesions.

Endometrial macrophages are thought to play important roles in repair and remodelling within the eutopic endometrium (Critchley *et al.*, 2001; Thiruchelvam *et al.*, 2013). Evidence from a mouse model of menstruation (utilised to generate 'menses-like' tissue in our model of endometriosis), identified an influx of Ly6C^{hi} monocytes which differentiated into macrophages during the repair phase of the menstrual cycle (Cousins *et al.*, 2016). Endometrial macrophages are also implicated

in regulating gland remodelling and angiogenesis by production of vascular endothelial growth factor (VEGF) (Sharkey *et al.*, 2000; Garry *et al.*, 2010). We know that macrophages are important for angiogenesis in endometriosis lesions (Bacci *et al.*, 2009), but the macrophage population responsible for this is unknown. I would hypothesise that due to the reparative roles macrophages are thought to play in the endometrium, endometrial-derived macrophages may play similar roles within the endometriosis lesion microenvironment, which could exacerbate disease by perpetuating the survival and health of ectopic endometrial tissue in the peritoneal cavity.

Using adoptive transfer experiments, I demonstrated that LPM infiltrate lesions whilst SPM do not. In endometriosis lesions, LPM formed discrete clusters of cells, suggesting they may be fulfilling localised functions within specific areas of the lesion. LPM are typically associated with homeostatic, tissue reparative roles during homeostasis in the peritoneal cavity, however it is unclear how these cells may behave in the microenvironment of endometriosis lesions (Ghosn *et al.*, 2010). In a mouse model of sterile liver injury, LPM infiltrated damaged tissue, where they adopted a 'pro-reparative' phenotype and fulfilled wound healing functions such as clearance of necrotic cells (Wang and Kubes, 2016). Due to the fact that LPM infiltrate lesions, I would hypothesise that this macrophage population exacerbates disease, due to their wound healing and tissue homeostatic roles and proven ability to infiltrate peritoneal organs, however this is currently unknown. SPM are associated with inflammation and clearing pathogens and foreign material in the peritoneal cavity (Ghosn *et al.*, 2010), thus, their inability to infiltrate lesions may contribute to the inefficient clearance of endometrial tissue, again, contributing to disease.

Using immunofluorescence, I demonstrated a high abundance of Ly6C^{hi} monocytes within endometriosis lesions as well as Ly6C^{hi} F4/80^{hi} monocyte-derived macrophages. Recruited macrophages are therefore largely derived from LPM and monocyte-derived macrophages. Currently do not know whether the Ly6C+ cells are a transient population that becomes educated within the lesion. Increasingly, studies are shedding light into the roles of monocytes and their dynamics in disease. For example, in atherosclerosis, Ly6C^{hi} monocytes migrate to diseased tissue where they

remain in a monocytic state, undergoing proliferation and having 'stem cell-like' properties (Lin *et al.*, 2019). It thus cannot be ruled out that Ly6C^{hi} monocytes may play distinct roles in endometriosis. My studies in CCL2 null mice that exhibit modestly decreased monocytes and monocytes derived macrophages with evidence of increased lesion number could support this concept. However, to my knowledge there have been no studies that have investigated this. Monocyte-derived macrophages have been shown, in a number of pre-clinical mouse models of cancer, to constitute a proportion of tumour-associated macrophages (Qian *et al.*, 2011; Madsen *et al.*, 2017; Zhu *et al.*, 2017). In a mouse model of breast cancer, Ly6C^{hi} monocytes are recruited to the tumour via CCL2 where they differentiate into macrophages and stimulate metastasis (Qian *et al.*, 2011). The tumour micro-environment is known to educate monocyte-derived macrophages such that they become 'pro-disease' upon incorporation into the tumour (Wu and Zheng, 2012; Noy and Pollard, 2014). Thus, in disease state, the microenvironment of pathological tissue has the potential to modulate monocyte-derived macrophage recruitment, phenotype and function. In endometriosis we don't know the role that monocyte-derived macrophages are fulfilling within the lesion microenvironment or the phenotype of these cells. I would hypothesise however, that local cytokines, chemokines and growth factors within the lesion microenvironment could modulate recruited Ly6C^{hi} monocytes such that they play a 'pro-disease' role in endometriosis. In my data, I demonstrated that Ly6C^{hi} F4/80^{hi} monocyte-derived macrophages were largely localised in clusters across lesions, suggesting that they may be playing roles within distinct areas of the pathological tissue.

Overall, I demonstrated that endometriosis lesion resident macrophages have multiple origins and are derived from donor endometrial tissue as well as recruited LPM and Ly6C^{hi} monocytes which differentiate (at least partly) into Ly6C^{hi} F4/80^{hi} macrophages.

6.1.3 CCR2 is important for accumulation of F4/80+ macrophages in lesions but the CCR2/CCL2 signalling pathway is redundant in the recruitment of Ly6C^{hi} monocytes

In chapter 4 I began to investigate the mechanism by which Ly6C^{hi} monocytes as well as peritoneal macrophage populations are recruited to endometriosis lesions. Using flow cytometry, I was able to characterise that mice with endometriosis had ~3 fold more CCR2+ peritoneal macrophages than sham controls and that 53% of lesion macrophages express the chemokine receptor CCR2. As the CCL2/CCR2 chemokine pathway is the classical pathway by which Ly6C^{hi} monocytes are recruited to tissues (Wynn, Chawla and Pollard, 2013), I utilised CCR2 and CCL2 knockout mice to decipher the effect of blocking this recruitment pathway on the development of endometriosis lesions in our model. Surprisingly, CCR2 knockout mice with endometriosis had a significant influx of Ly6C^{hi} monocytes into the peritoneal cavity and developed a similar number of lesions to WT. CCL2 knockout mice with endometriosis also had a similar number of peritoneal Ly6C^{hi} monocytes to WT but developed more lesions, however this may have been attributed to a significant increase in general leukocyte number in the peritoneal cavity. Importantly, endometriosis lesions from CCR2 and CCL2 knockout mice had a similar number of Ly6C^{hi} monocytes to WT lesions at immunofluorescent analysis, but CCR2 knockout lesions had less F4/80+ macrophages. Together, this data provides evidence that endometriosis lesions produce a chemotactic signal for Ly6C^{hi} monocytes which causes their mobilisation from the bone marrow and subsequent extravasation into lesions. In our model, recruitment of Ly6C^{hi} monocytes can occur independent of the CCR2/CCL2 chemokine pathway usually required for their chemotaxis (Wynn, Chawla and Pollard, 2013), suggesting that this pathway is redundant in endometriosis. CCR2 however appears to be important for the accumulation of F4/80+ macrophages in lesions, as in the absence of CCR2, the number of F4/80+ cells in lesions was reduced. The fact that I didn't see a change in lesion F4/80+ macrophages in the CCL2 knockout animals suggests that macrophage chemotaxis is not completely reliant on the CCL2 ligand but is reliant on signalling through CCR2.

Overall, this data provides insight into the role of the CCR2/CCL2 recruitment pathway in endometriosis and demonstrates that whilst this axis is redundant in the recruitment of Ly6C^{hi} monocytes, a subset of F4/80+ macrophages relies on CCR2 for recruitment to lesions. I also showed that, in our model, the presence of endometriosis lesions in the peritoneal cavity causes the mobilisation of Ly6C^{hi} monocytes from the bone marrow and subsequent infiltration into lesions.

Blocking the recruitment of TAMs is a therapeutic mechanism that has been investigated in a number of cancer studies and has been shown to provide some clinical efficacy in pre-clinical mouse models (Qian *et al.*, 2011; Svensson *et al.*, 2015; Lim *et al.*, 2016). A phase 1B clinical trial in pancreatic adenocarcinoma patients utilised a CCR2 inhibitor to block the recruitment of monocyte-derived TAMs, which was dosed in combination with chemotherapy (PF-04136309). This feasibility trial reported that peripheral monocytes were reduced in the patients after CCR2 inhibitor treatment, thus diminishing the pool of monocytes available to be recruited to tumours. Analysis of tumour biopsies revealed that recruitment of monocyte-derived TAMs was significantly reduced (assessed by FACS and qPCR). Further trials are required to determine the clinical efficacy of treatment, but this trial demonstrated that inhibiting the recruitment of TAMs in humans is possible and well tollerated by patients (Wang-Gillam A.,*et al.*, 2015). Thus, understanding the pathways which regulate the recruitment of lesion resident monocyte/macrophage populations could provide the opportunity to therapeutically intervene with this recruitment process, to demontrate clinical benefit. Currently, we have very little knowledge about the recruitment pathways active in endometriosis. However, in this thesis I have demonstrated for the first time, that the CCR2-CCL pathway is redundant for recruitment of monocytes but involved in chemotaxis of mature macrophages. This provides the basis for futher studies investigating other monocytes / macrophages recruitment and differentiation factors in endometriosis

6.1.4 Lesion macrophages are a phenotypically heterogeneous population

In chapter 5 I interrogated the transcriptomic profile of CD45+ leukocytes from donor endometrial tissue, endometriosis lesions as well as peritoneal cells from endometriosis and sham mice. Single cell RNA sequencing revealed that

endometriosis lesion *Csf1r+ Adgre1+* macrophages had a more similar transcriptomic profile to eutopic endometrial macrophages than to peritoneal macrophages but up-regulated a number of novel genes which were specific to the endometriosis disease state. Lesion macrophages were transcriptomically heterogeneous. I identified five sub-populations based on transcriptional profile, one population of which up-regulated many proliferation markers including *Mki67*, identifying a population of macrophages that have the intrinsic ability to proliferate *in situ*. Importantly, in this chapter I also demonstrated that lesion macrophages can be stratified into sub-populations based on cell surface marker expression and flow cytometry analysis, providing proof that sub-populations are also present at the protein level. This data provides an insight into the thus far unappreciated heterogeneity of endometriosis lesion macrophages and sheds light on macrophage phenotypic 'education' within the lesion microenvironment. The fact that endometriosis lesion macrophages have a number of uniquely up-regulated genes suggests that macrophages are being educated in the endometriosis lesion microenvironment, akin to tumour associated macrophages once they become incorporated into tumours (Cassetta *et al.,* 2019). I hypothesise that this phenotypic switching may be critical to the pro-disease roles we know macrophages play within the lesion microenvironment (Bacci *et al.,* 2009; Greaves *et al.,* 2015; Forster *et al*., 2019). Macrophages may therefore respond to local tissue cues in the lesion environment which cause their subsequent development into 'pathology-associated' cells.

In tumours it is known that multiple macrophage populations exist which play differential roles in disease, up-regulating defined surface markers and exhibiting differential gene expression patterns (Noy and Pollard, 2014). It is possible therefore that the multiple transcriptionally diverse macrophage populations I identified within endometriosis lesions could be playing discrete roles in the pathology of endometriosis. Gene ontology analysis revealed insight into the functions that the five transcriptionally diverse macrophage populations I identified may be playing within lesions. Cluster 1 up-regulated genes assocaited with neurogenesis and stimilating the proliferation and survival of stromal cells, functions already known to be fulfilled by lesion resident macrophages to exacerbate disease (Chan *et al.,* 2017;

Yang *et al.,* 2017; Forster *et al*., 2019). Cluster 2 up-regulated a number of genes involved in neutrophil chemotaxis, as well as genes involved in stimulating the proliferation of fibroblasts. The role of neutrophils in endometriosis remains undefined, but my data implies that macrophage-neutrophil signalling occurs within lesions. Fibroblasts have the potential to play a pro-disease role within lesions due to their ability to regulate the extracellular matrix and mediate wound healing responses, however this is currently unknown (McAnulty, 2007). This data does indicate that macrophage-fibroblast cross-talk may be implicated in endometriosis. Cluster 3 up-regulated genes associated with modulating immune responses, apoptotic activity and recruitment of B cells. I would hypothesise that this macrophage population may play a role in mediating the immune response within endometriosis lesions such that ectopic endometrial tissue is able to escape immune clearance, a key hallmark of endometriosis (Burney and Giudice, 2012), however this is unknown. In my single cell RNA sequencing data I identified a population of lesion resident B cells, but the role of this population is unknown. This data does suggest, however, that lesion macrophages are involved in the chemotaxis of B cells to lesions. Macrophage cluster 4 up-regulated genes involved in response to cytokine stimuli and notably up-regulated a number of the *S100* genes proven to have an intricate role in cancer pathophysiology (Bresnick, Weber and Zimmer, 2015), as well as the *Lsp1* gene shown to be involved in preventing T cell recruitment to tumours (Hwang *et al.,* 2015). The last cluster, cluster 5, was defined by its ability to proliferate within lesions.

What these ontology analyses highlight is that macrophage function is likely to be diverse within endometriosis lesions, and involves reciprocal signalling between macrophages and a number of different cell types, such as neutrophils, B cells, T cells, fibroblasts and stromal cells. Underpinning the roles these macrophages are playing and how macrophages are modulated to fulfil these roles in endometriosis, could facilitate the development of specific treatments which target macrophage sub-populations known to play roles in pathology linked to adverse clinical outcomes.

6.2 Summary

Overall, in this thesis I have provided novel insight into the biology of macrophage populations in our mouse model of endometriosis and revealed previously unappreciated complexity in macrophage ontogeny and transcriptomic profile within endometriosis lesions.

I have demonstrated that endometriosis lesions produce a chemotactic signal for Ly6C^{hi} monocytes that differentiate into macrophages within lesions, and this recruitment can occur independent of the classical CCR2/CCL2 pathway, but my results suggest that recruitment of a population of F4/80+ macrophages to lesions relies on CCR2. Importantly, I characterised that lesion macrophages are heterogeneous in both origin and transcriptional profile. Lesion macrophages are comprised of endometrial macrophages derived from donor endometrial tissue as well as recruited of LPM and monocyte-derived macrophages. A sub-population of lesion macrophages are also maintained by *in situ* proliferation. Within lesions, five discrete macrophage populations exist which have unique transcriptional profiles, and gene ontology analysis suggests that these macrophage sub-populations could play differential roles in pathology and interact with a number of different cell types within lesions. In *figure 6.1* I summarise my findings from this thesis.

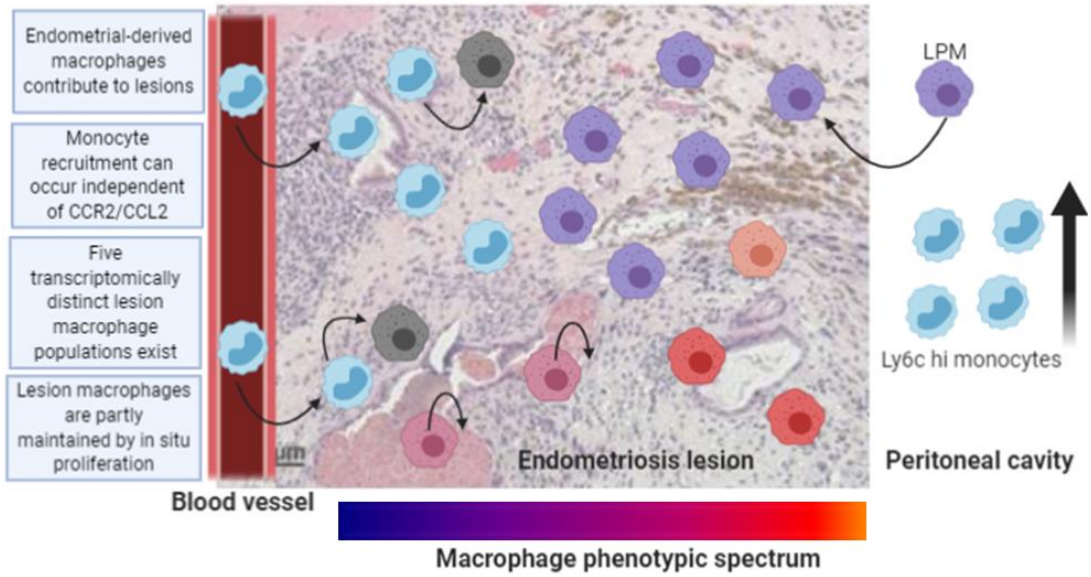


Figure 6.1 Thesis summary

In endometriosis lesions, macrophages are derived from endometrial-derived macrophages, as well as recruited populations which consist of large peritoneal macrophages (LPM) and monocyte-derived macrophages. In our mouse model, mice with endometriosis have an influx of Ly6C^{hi} monocytes into the peritoneal cavity. Ly6C+ monocytes infiltrate lesions where they differentiate into Ly6C+ F4/80+ macrophages and this recruitment can occur independent of the CCR2/CCL2 chemokine axis. Recruitment of F4/80+ macrophages however at least partly relies on CCR2 signalling. At single cell RNA sequencing analysis, lesion macrophage form five transcriptionally unique populations and a sub-set of these up-regulate the proliferation marker Mki67, identifying their ability to expand by in situ proliferation. Overall, this thesis provides evidence of previously unappreciated endometriosis lesion macrophage heterogeneity, in both origin and transcriptomic profile.

Chapter 7 - Future work and impact of thesis

In this thesis I identified a previously unappreciated heterogeneity in lesion macrophages, both in their origin and transcriptomic profile. This has implications on the interpretation of previous research which has aimed to investigate endometriosis lesion macrophage phenotype, as these studies largely utilised immunohistochemical techniques to define phenotypic markers expressed by lesion macrophages (Bacci *et al.*, 2009; Smith *et al.*, 2012; Johan *et al.*, 2019b; Yamada *et al.*, 2019). Whilst these studies have provided an important insight into lesion macrophage phenotype, it is possible that multiple macrophage populations exist not only in our mouse model, but in other pre-clinical models of endometriosis. When comparing our results and other models of endometriosis, it must be noted that these models do not truly recapitulate the chronic aetiology of a lesion, and represent a more transient and acute disease state. Comparison of lesion macrophages across models in comparison to disease in humans is thus a fundamental bridge for understanding the true dynamics of macrophage phenotype in endometriosis.

Moreover, functional experiments aiming to identify the roles of macrophages in endometriosis may have assigned these roles to generalised 'endometriosis-associated macrophages' when this role is actually carried out by a distinct sub-population of macrophages within the lesion. Defining the roles that macrophages with different origins and transcriptional profiles play within the disease setting is an important area of future research. Studies aiming to discern the roles of these populations could include depletion of macrophages with a different origin, or with a unique transcriptional profile within lesions, or adoptive transfer of said populations into the peritoneal cavity (gain of function), to discern the effect on lesion development. These studies are an important step to assess the potential efficacy of targeting macrophage populations therapeutically.

In this thesis I identified a unique genetic profile for endometriosis lesion macrophages. The roles of genes exhibiting up-regulation by endometriosis lesion macrophages should be investigated in functional studies in future research to ascertain if these genes could play central roles in disease. Studies may include the use of knockout mouse or cell lines which discern the effect of negating this gene in the development of pathology and its associated symptoms. Thus, this work should

aim to elucidate the function of these genes and determine the suitability of candidates as therapeutic targets for women with endometriosis. Importantly, we can learn a lot about these targets from studies in other therapeutic areas. A prime example of this is *Gas6*, which was highly up-regulated by the largest lesion macrophage population. We already know from cancer studies that this gene is implicated in stimulating pathology in the tumour environment by promoting the growth and survival of cancer cells (Hutterer *et al.*, 2008; Loges *et al.*, 2010; Korshunov, 2012). We can utilise this research to generate hypotheses around how, for example *Gas6*, may be involved in endometriosis pathophysiology. Future research should therefore utilise studies within fields, especially the cancer field, which draws a lot of parallels to endometriosis (Wiegand *et al.*, 2010; Heidemann *et al.*, 2014), and employ functional studies (knock out models, *in vitro* analyses) to discern: 1) whether or not genes up-regulated by lesion macrophages are important in the development or exacerbation of pathology and 2) the suitability of these targets for providing clinical benefit whilst also promoting the long term health of the patient.

I also began to investigate the recruitment pathways active in disease which are responsible for the recruitment of monocytes/macrophages to endometriosis lesions. A key difficulty in these studies was the potential for chemokines/cytokines to compensate for the knockout of CCR2 or CCL2, making conclusions on the importance of this pathway in the disease setting somewhat difficult. We know that a number of cytokines/chemokines are up-regulated in the peritoneal cavity of women with disease, but which of these is required for monocyte/macrophage recruitment is currently unknown (Jørgensen *et al.*, 2017). Future research should focus on assessing how, in the absence of CCR2 or CCL2, monocytes are still able to be mobilise from the bone marrow and be recruited to endometriosis lesions. Thus, which chemokines/cytokines are up-regulated in lesion tissue and in the peritoneal cavity in the absence of CCR2 or CCL2, and whether or not this is different to disease state where CCR2/CCL2 are present. It would be very interesting to see if blocking this recruitment can have an effect on the development of endometriosis lesions, and would thus give insight into the role of Ly6C^{hi} monocytes in disease. Future research could also utilise lineage tracing studies to define which F4/80+

macrophage population is being recruited by CCR2, and use functional studies to determine the role that this recruited population is fulfilling within the endometriosis lesion microenvironment. Overall, further work is required to understand the mechanism by which monocytes/macrophages are recruited to lesions, and the importance of this recruitment in the development of pathology.

To conclude, exploring and exploiting the heterogeneity and recruitment of macrophages into endometriosis lesions could lead to the development of therapeutic treatments which have the ability to specifically target macrophages with distinct roles in disease development. These treatments may be able to provide clinical benefit whilst avoiding unspecific targeting of macrophage populations throughout the body, thus promoting the long term health of the patient. Whilst realisation of these treatments could take many years, this thesis provides a platform for further research into macrophage targeted therapies for women with endometriosis.

Chapter 8 - Appendix

Appendix 1. Significant differentially expressed genes in Csf1r+ Itgam+ Adgre1+ macrophage sub-populations in mouse endometriosis lesions as determined by single cell RNA sequencing

∗; P < 0.1, ∗∗; P < 0.05, ∗∗∗; P < 0.01 and ****; P < 0.001. P values were adjusted using the Benjamini-Hochberg correction for multiple tests by Loupe Cell Browser 3.0.1 software.

Cluster 1		
Gene Name	**Log2 fold change**	**P value**
Gas6	+3.23	****
Ccl8	+3.01	****
Igfbp4	+2.68	****
Cbr2	+2.58	****
Wwp1	+2.47	****
Stab1	+2.36	****
Folr2	+2.34	****
Maf	+2.24	****
Sepp1	+2.13	****
Mrc1	+2.13	****
F13al	+2.03	****
Dab2	+2.00	****
Clec10a	+1.90	****
Fcgrt	+1.84	****
Serinc3	+1.68	****
Igf1	+1.65	****
Fcrls	+1.52	****
Abca1	+1.52	****
Pf4	+1.49	****
Ly6e	+1.48	****
Nrp1	+1.48	****
Cfh	+1.46	****
C5ar1	+1.45	****
Cltc	+1.36	****
Ms4a7	+1.34	****
Itm2b	+1.31	****
Zfp36l1	+1.30	****
Marcks	+1.29	****
Ehd4	+1.27	****
C1qc	+1.27	****
C4b	+1.27	****
Apoe	+1.24	****
Fcgr2b	+1.23	****
Ninj1	+1.23	****
Serpinb6a	+1.22	****
Ms4a6b	+1.21	****
Mafb	+1.20	****
Mgl2	+1.20	****

C1qa	+1.19	****
Csf1r	+1.18	****
Txnip	+1.16	****
Lgmn	+1.15	****
Ctsc	+1.12	****
Adgre1	+1.10	****
Ednrb	+1.05	****
Snx2	+1.03	****
Trf	+1.03	****
Glul	+1.01	****
C3ar1	+1.00	****
Grn	+0.97	****
Pltp	+0.97	***
Bst2	+0.94	***
C1qb	+0.94	***
Ctsb	+0.93	***
Clec4a1	+0.91	***
Unc93b1	+0.90	***
Fcgr3	+0.85	***
Cd81	+0.85	***
Wfdc17	+0.81	***
Lst1	+0.80	**
Snx5	+0.77	**
Picalm	+0.77	**
Tsc22d3	+0.76	**
Asah1	+0.76	**
Timp2	+0.72	**
Aldh2	+0.71	**
Blvrb	+0.70	**
Ptpn18	+0.67	**
Ly86	+0.67	**
Rgs10	+0.67	**
Snx3	+0.65	*
Lamp1	+0.65	*
Atp2b1	+0.64	*
Laptm4a	+0.64	*
Hexa	+0.63	*
Aif1	+0.62	*
Tmem176b	+0.62	*
Pld4	+0.62	*
Vsir	+0.61	*

Cluster 2		
Gene Name	**Log2 fold change**	**P value**
Mmp12	+6.27	****
Alas1	+4.96	****
Cxcl3	+3.48	****
Prdx1	+2.71	****
Lpl	+2.70	****
Esd	+2.56	****
Cd9	+2.56	****
Gpnmb	+2.55	****
Cstb	+2.40	****
Spp1	+2.28	****
Lgals3	+2.26	****
Clec4d	+2.17	****
Sod2	+2.16	****
Fabp5	+2.16	****
Capg	+2.10	****
Pgam1	+1.99	****
Fn1	+1.89	****
Anxal	+1.82	****
Itgb2	+1.80	****
Txn1	+1.79	****
Aldoa	+1.78	***
Pgd	+1.72	***
Fth1	+1.72	***
Anxa4	+1.70	***
Lilrb4a	+1.70	***
Pkm	+1.65	***
Lyz1	+1.64	**
Cxcl2	+1.60	**
Clec4n	+1.60	***
Fabp4	+1.57	**
Creg1	+1.56	**
Mif	+1.55	**
Tkt	+1.44	**
Gngt2	+1.42	**
Aprt	+1.40	**
Rnh1	+1.38	**
Card19	+1.35	**
Ccl9	+1.34	*
Cybb	+1.34	*
S100a4	+1.30	*
Taldo1	+1.30	*
Lipa	+1.29	*
Eno1	+1.25	*

Cluster 3		
Gene Name	**Log2 fold change**	**P value**
Alox15	+5.45	****
Prg4	+5.42	****
Cxcl13	+5.15	****
Saa3	+4.96	****
Prtn3	+4.75	****
Spi	+4.61	****
Itcam2	+4.59	****
Padi4	+4.32	****
Lrg1	+3.96	****
Mcemp1	+3.81	****
Cd5l	+3.79	****
Thbs1	+3.43	****
Fabp4	+3.17	****
Fn1	+3.09	****
Itga6	+2.90	****
Emilin2	+2.86	****
Ltc4s	+2.81	****
Smpdl3a	+2.54	***
Ednrb	+2.20	**
Klf2	+2.17	**

Cluster 4		
Gene Name	**Log2 fold change**	**P value**
Retnla	+3.23	****
Lsp1	+2.42	****
Tmsb10	+1.78	****
Id2	+1.40	****
S100a11	+1.31	****
Fabp4	+1.22	**
Cd52	+1.10	****
Cxcl2	+1.07	**
Lyz1	+1.04	**
S100a4	+0.97	***
Clec4b1	+0.95	***
S100a6	+0.95	***
Mcemp1	+0.92	**
Gm8730	+0.90	**
Crip1	+0.87	**
Gm2a	+0.84	**
Srgn	+0.83	**
Nfkbia	+0.83	**
Fn1	+0.82	**
Rplp0	+0.79	**
H2-DMb1	+0.79	**

Cycs	+0.78	**
Ccr2	+0.76	**
Fxyd5	+0.75	**
Thbs1	+0.75	*
Anxa2	+0.74	*
Actg1	+0.72	*
Rps7	+0.72	*
Coro1a	+0.69	*

Cluster 5		
Gene Name	**Log2 fold change**	**P value**
2810417H13Rik	+6.50	****
Top2a	+6.30	****
Birc5	+6.18	****
Stmn1	+4.77	****
Tmpo	+3.35	****
Tuba1b	+2.74	****
Tubb5	+2.67	****
Pcna	+2.59	****
Ube2s	+2.40	****
Slpbp	+2.37	****
H2afv	+2.23	****
H2afz	+2.10	****
Anp32bm	+2.08	****
Nucks1	+2.06	****
Dek	+2.00	****
Ptma	+1.78	***
Ranbp1	+1.59	**
Nap111	+1.59	**
Ran	+1.55	**
Srsf3	+1.37	*

Appendix 2. Significant differentially expressed genes in Csf1r+ Itgam+ Adgre1+ macrophages in mouse decidualised endometrial tissue, peritoneal lavage fluid and endometriosis lesions as determined by single cell RNA sequencing

∗; P < 0.1, ∗∗; P < 0.05, ∗∗∗; P < 0.01 and ****; P < 0.001. P values were adjusted using the Benjamini-Hochberg correction for multiple tests by Loupe Cell Browser 3.0.1 software.

Endometrial Tissue		
Gene Name	**Log2 fold change**	**P value**
Il1b	+4.65	****
Ly6c2	+4.62	****
Ccl4	+4.35	****
Hspa1a	+3.90	****
Ms4a4c	+3.68	****
Gatm	+3.00	****
Rgs1	+3.12	****
Isg15	+3.04	****
Cxcl2	+3.25	****
Cd83	+2.97	****
Jun	+2.60	****
Stmn1	+2.59	****
Plac8	+2.58	****
Ms4a6b	+2.50	****
Dnajb1	+2.58	****
Mmp12	+2.35	****
Clec4n	+2.22	****
Fos	+2.22	****
Plbd1	+2.22	****
Hsp90aa1	+2.04	****
Hspa1b	+1.94	****
Pim1	+1.84	****
Fcgr1	+1.83	****
Hmox1	+1.81	****
Cxcl16	+1.80	****
Hspd1	+1.77	****
Efhd2	+1.76	****
Hspa8	+1.73	****
Lgals1	+1.72	****
Ms4a6d	+1.71	****
Ms4a6c	+1.65	****
Junb	+1.64	****
Tubb5	+1.62	****
Coro1a	+1.61	****
Actg1	+1.59	****
Litaf	+1.59	****
Btg1	+1.59	****

Lst1	+1.57	****
Gdi2	+1.56	****
Ube2s	+1.56	****
Dnaja1	+1.56	****
Clec4a2	+1.55	****
Slc3a2	+1.53	****
Ptma	+1.49	****
Hspe1	+1.48	****
AF251705	+1.48	****
Nfkbia	+1.47	****
Fth1	+1.44	****
Jund	+1.44	****
Lcp1	+1.42	****
Tpm4	+1.41	****
Ubc	+1.38	****
Tpd52	+1.38	****
Cstb	+1.38	****
Aif1	+1.36	****
Ran	+1.36	****
Tmsb10	+1.34	****
Fcrls	+1.34	****
H2afy	+1.34	****
Lilrb4a	+1.32	****
Atp5b	+1.28	****
Mif	+1.25	****
Tuba1b	+1.25	****
Dusp1	+1.25	****
Pgam1	+1.25	****
Prdx1	+1.24	****
Hexb	+1.24	****
Rbm3	+1.23	****
Lgmn	+1.22	****
Tgfbi	+1.21	****
Polr2l	+1.20	****
Lgals3	+1.19	****
Pkm	+1.18	****
Set	+1.18	****
Tpi1	+1.17	****
Gm2a	+1.17	****
Serinc3	+1.16	****
H2afz	+1.16	****
Ms4a7	+1.16	****
Slc25a5	+1.15	****
Cycs	+1.12	***
Snx5	+1.12	***
Cfl1	+1.12	***

Hnrnpa2b1	+1.11	***
Marcksl1	+1.11	***
Ly86	+1.10	***
Sat1	+1.10	***
Anxa5	+1.10	***
Clec12a	+1.10	***
Myl12a	+1.10	***
Dab2	+1.10	***
Hcls1	+1.08	***
H2-DMb1	+1.07	***
Atp6ap2	+1.06	***
Ctsc	+1.06	***
Hsp90ab1	+1.05	***
Bst2	+1.05	***
Eif4a1	+1.04	***
Hnrnpa3	+1.04	***
Clec4a3	+1.04	***
H2-Eb1	+1.03	***
Ppia	+1.02	***
Ywhah	+1.02	***
S100a11	+1.00	***
Nmp1	+0.99	***
Ccr2	+1.00	***
Psma1	+0.98	***
Cd209a	+0.98	***
H3f3b	+0.97	***
Srsf3	+0.97	***
Ranbp1	+0.97	***
Cnbp	+0.95	***
Psma3	+0.95	***
Ncl	+0.95	***
Ybx1	+0.95	***
Mpeg1	+0.95	***
Snx2	+0.95	***
H2-Ab1	+0.94	***
Picalm	+0.94	***
H2-Aa	+0.94	***
Syngr2	+0.94	***
Rnh1	+0.94	**
Vdac2	+0.94	**
Anp32b	+0.93	**
Ddx5	+0.93	**
Chmp4b	+0.93	**
Psma4	+0.92	**
Arl6ip1	+0.92	**
Abracl	+0.92	**

Cd74	+0.92	**
Atp5c1	+0.91	**
Tkt	+0.89	**
Akr1a1	+0.88	**
Cd14	+0.87	**
Sirpa	+0.86	**
Tmsb4x	+0.86	**
Cct8	+0.86	**
Cct5	+0.86	**
Cd52	+0.84	**
Srsf2	+0.84	**
Arpc3	+0.84	**
Ube2d3	+0.83	**
Esd	+0.84	**
Ifi27l2a	+0.83	**
Actr3	+0.82	**
Lyn	+0.82	**
Aprc4	+0.82	**
Aldoa	+0.81	**
S100a9	+0.81	**
Ifi30	+0.81	**
Eif2s2	+0.81	**
Cct4	+0.81	**
Calm3	+0.80	**
Hnrnpk	+0.78	**
Eif5a	+0.78	**
Calm1	+0.78	**
Gapdh	+0.77	**
Arhgdia	+0.77	**
Atp5f1	+0.77	**
Spp1	+0.77	**
Hrnrpa1	+0.76	**
Psmb6	+0.76	**
Rab7	+0.76	*
Psme1	+0.76	*
Cox7b	+0.76	*
Taldo1	+0.76	*
Capza2	+0.75	*
Nme1	+0.75	*
Ptprc	+0.74	*
Arpc2	+0.74	*
Cdc42	+0.74	*
Actb	+0.74	*
Tgfb1	+0.74	*
Hspa5	+0.73	*
Ywhae	+0.73	*

Pcbp1	+0.73	*
Anxa2	+0.73	*
Rac1	+0.72	*
Vim	+0.72	*
Rplp0	+0.70	*
Serbp1	+0.69	*
Clta	+0.69	*
Btf3	+0.69	*
H3f3a	+0.69	*
Pfn1	+0.68	*

Peritoneal Lavage Fluid		
Gene Name	**Log2 fold change**	**P value**
Cxcl13	+5.57	****
Slpi	+5.51	****
Alox15	+5.48	****
Padi4	+5.18	****
Prtn3	+4.78	****
Icam2	+4.75	****
Fcna	+4.43	****
Prg4	+4.36	****
Saa3	+4.01	****
Lrg1	+3.94	****
Cd5l	+3.90	****
Fabp4	+3.48	****
Fn1	+3.29	****
Ltc4s	+3.16	****
Hgsnat	+3.04	****
Mcemp1	+2.93	****
F10	+2.89	****
Emilin2	+2.89	****
Itga6	+2.88	****
Klf2	+2.87	****
Ccl24	+2.87	****
Lyz1	+2.76	****
C4b	+2.68	****
Ecm1	+2.65	****
Smpdl3a	+2.48	****
Ednrb	+2.35	****
Hp	+2.27	****
Cd37	+2.08	****
Nupr1	+1.96	****
Dpep2	+1.92	****
Ifitm6	+1.88	****
PycarLyz2d	+1.85	****
Thbs1	+1.81	****

Tcn2	+1.80	****
Tmcc1	+1.74	****
Fam46a	+1.71	****
Timp2	+1.67	****
Trf	+1.64	****
Msr1	+1.64	****
Gngt2	+1.56	****
Cfp	+1.56	****
Wfdc17	+1.51	****
Selplg	+1.51	****
Mgst1	+1.48	****
Itgb2	+1.48	****
Prdx5	+1.41	****
Tagln2	+1.37	****
Tln1	+1.34	****
Idh1	+1.29	****
Ccl6	+1.28	****
Itgam	+1.27	****
Man2b1	+1.25	****
Fxyd5	+1.23	****
Gpx1	+1.20	****
Adgre5	+1.18	****
Ssr4	+1.12	****
S100a1	+1.11	****
Pf4	+1.08	****
Prr13	+1.05	****
Gda	+1.04	****
Bsg	+1.01	****
Comt	+1.00	****
Tmed3	+1.00	****
Cfh	+0.99	****
Aldh2	+0.99	****
Lamtor4	+0.98	****
Apoe	+0.98	****
Uba52	+0.96	****
Gpx4	+0.92	***
Ninj1	+0.85	***
Hmha1	+0.84	***
Crip1	+0.84	***
Srgn	+0.81	***
Ccdc12	+0.78	***
Cd9	+0.78	***
Ppib	+0.76	***
C1qc	+0.75	**
Gm10076	+0.73	**
Ifitm3	+0.72	**

Gmfg	+0.70	**
Rps19	+0.68	**
Shisa5	+0.66	**
Dbi	+0.65	**
Emp3	+0.65	**
Mt-Nd1	+0.64	**
Sdf2l1	+0.64	**
C1qa	+0.63	**
Ddost	+0.63	**
H2-D1	+0.63	**
1110008F13Rik	+0.63	**
Mrpl33	+0.62	**
Rps8	+0.62	**
Itgb1	+0.62	**
Ahnak	+0.62	**
Sdc3	+0.61	**
Cyba	+0.61	**
Cybb	+0.59	**
Rpn2	+0.56	*
Rpl8	+0.54	*
Rpl36a	+0.54	*
Sec11c	+0.53	*
Rpl4	+0.53	*
Rpl10-ps3	+0.53	*
Alox5ap	+0.52	*
Rpsa	+0.52	*
Eno1	+0.52	*
Rpl2mt-Cytb3	+0.51	*
Dad1	+0.51	*
Uqcr11	+0.51	*

Endometriosis Lesions		
Gene Name	**Log2 fold change**	**P value**
Ccl8	+5.84	****
Gas6	+3.99	****
Mrc1	+3.61	****
Spp1	+3.40	****
Ms4a7	+3.22	****
Marcks	+3.17	****
Wwp1	+3.12	****
Abca1	+3.04	****
Cbr2	+3.00	****
Maf	+2.92	****
Cd63	+2.83	****
Stab1	+2.75	****
Fcrls	+2.56	****

Clec10a	+2.46	****
Clec4a2	+2.43	****
Hspa1b	+2.35	****
Sepp1	+2.29	****
Clec12a	+2.28	****
Cltc	+2.22	****
Dab2	+2.20	****
F13a1	+2.13	****
Mgl2	+2.15	****
Igf1	+2.11	****
Folr2	+2.11	****
Ehd4	+2.08	****
Zfp36l1	+1.99	****
Clec4a1	+1.93	****
Mt-Nd4l	+1.93	****
Clec4n	+1.93	****
Lst1	+1.90	****
Sirpa	+1.90	****
Clec4a3	+1.89	****
AF251705	+1.90	****
Serinc3	+1.85	****
Trem2	+1.82	****
Picalm	+1.82	****
Anxa5	+1.81	****
Ctsc	+1.81	****
Qk	+1.81	****
Fos	+1.76	****
Fcgr2b	+1.73	****
Nrp1	+1.71	****
Aif1	+1.69	****
Ccr2	+1.66	****
Lgmn	+1.62	****
Mmp12	+2.03	****
Itm2b	+1.58	****
BC005537	+1.58	****
S100a9	+1.58	****
Lgals1	+1.56	****
S100a8	+1.55	****
Ms4a6b	+1.54	****
Igfbp4	+1.53	****
Slc6a6	+1.53	****
Kctd12	+1.51	****
Atp2b1	+1.48	****
Cxcl16	+1.42	****
Txnip	+1.38	****
Gm42418	+1.40	****

Mpeg1	+1.38	****
Fcgrt	+1.35	****
Ctsb	+1.34	****
Serpinb6a	+1.35	****
Ctsl	+1.32	****
Snx5	+1.32	****
Psap	+1.27	****
Lgals3	+1.27	****
Tpd52	+1.25	****
Klf6	+1.25	****
Jun	+1.23	****
C3ar1	+1.21	****
Cyth4	+1.15	****
Sat1	+1.14	****
Actr2	+1.14	****
Prcp	+1.13	****
Pla2g7	+1.12	****
Ptprc	+1.10	****
Calm2	+1.10	****
Mbnl1	+1.09	****
Flt1	+1.08	****
Gm10116	+1.08	****
Cstb	+1.08	****
Gatm	+1.08	****
Cd83	+1.08	****
H2-Aa	+1.04	***
Cd74	+1.02	***
Fcgr1	+1.02	***
Mt1	+0.99	***
Rtn4	+0.97	***
H2-DMb1	+0.96	***
Zfp36l2	+0.96	***
Tsc22d3	+0.94	***
Efhd2	+0.95	***
Aplp2	+0.93	***
Lpl	+0.94	***
Sptssa	+0.92	***
Akr1a1	+0.91	***
Wnk1	+0.90	***
Rgs1	+0.90	***
Lcp1	+0.89	***
Prdx1	+0.89	***
Unc93b1	+0.87	***
Tmem106a	+0.86	***
Hmox1	+0.88	**
Celf2	+0.85	**

Capza2	+0.84	**
Arl6ip1	+0.83	**
Atp6ap2	+0.83	**
Morf4l1	+0.82	**
Cd47	+0.82	**
Dusp1	+0.82	**
Cd44	+0.82	**
Iqgap1	+0.79	**
Zeb2	+0.78	**
Gltp	+0.78	**
Slc3a2	+0.78	**
Canx	+0.77	**
Csf1r	+0.77	**
Ndufc2	+0.76	**
Cd81	+0.76	**
Reep5	+0.76	**
Ms4a6c	+0.76	**
Sdcbp	+0.75	**
Tmem176a	+0.75	**
Tmem176b	+0.74	**
C5ar1	+0.74	**
Hs-Dma	+0.73	**
Asah1	+0.73	**
Ddx5	+0.73	**
Dazap2	+0.73	**
Anax2	+0.70	**
Dnajb1	+0.70	**
Pltp	+0.69	**
Pld4	+0.68	*
Atp6v1g1	+0.67	*
Polr2l	+0.67	*
Ifngr1	+0.67	*
Ypel3	+0.67	*
Fcer1g	+0.66	*
Ptp4a2	+0.63	*
mt-Co1	+0.62	*
mt-Co2	+0.62	*
Tmsb4x	+0.61	*
Btg1	+0.61	*
Coro1a	+0.61	*
Vim	+0.61	*
Hsp90aa1	+0.61	*
Gm2a	+0.60	*
Actb	+0.60	*

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